The Effects of Morphine Treatment on Intracellular Signalling in the Young Rat

by
Anika Ayo McPhie

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

2005

UMI Number: 3187387

Copyright 2005 by
McPhie, Anika Ayo

All rights reserved.

UMI Microform 3187387
Copyright 2005 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

© 2005

ANIKA AYO MCPHIE

All Rights Reserved

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.

Date	Gordon Barr, Ph.D., Chair of Examining Committee
Date	Joseph Glick, Ph.D., Executive Officer

Jesus Angulo, Ph.D.

Gordon Barr, Ph.D.

Shirzad Jenab, Ph.D.

Vanya Quiñones-Jenab, Ph.D.

Regina Sullivan, Ph.D.
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

THE EFFECTS OF MORPHINE TREATMENT ON INTRACELLULAR SIGNALLING IN THE YOUNG RAT

by
Anika Ayo McPhie

Advisor: Professor Gordon Barr

The medicinal value and pleasurable effects of opiates have been known of for hundreds of years. Clinically, one major drawback of long-term opiate treatment is that it leads to tolerance and dependence. Many decades of research have gone into understanding these phenomena by studying the manifested behaviors in both human and animal models. A major finding has been that they occur and can be modeled in the young rodent. With the advancement of molecular techniques the IP_3-DAG and cAMP/AC signal transduction pathways have been shown to regulate and be regulated by the opiate system. However, the majority of this work has been done in the adult organism, despite the fact that a constant population of human infants is exposed to opiates. These neonates and fetuses experience both tolerance and withdrawal, the latter of which is linked to increased morbidity and later cognitive problems. Because the mechanisms of tolerance and dependence differ between infant (PD7-14) and older animals (PD21+), the current experiments were conducted to determine if there are ontogenetic interactions between opiates and the IP_3-DAG and cAMP/AC signal transduction systems. In the first set of experiments, the activity of the opioid receptors was pharmacologically blocked and the effects on withdrawal at PD7 were quantified. Results were similar to those found in the adult; the μ-opioid receptor played the

predominant role in modulating withdrawal. Next, immunocytochemistry was employed to determine the neuroanatomical location of the activated gene *c-fos* during opiate withdrawal at PD7. Once again the results were similar to those found in adult rodents; increased Fos-like immunoreactivity was found in similar brain regions. Finally, the interaction between morphine and protein kinases was explored in two age groups, PD7 and PD21. Unlike the adult literature, pharmacological blockade of PKA and PKC had no inhibitory effect on withdrawal or tolerance. In addition, overall changes in levels of PKA and PKC were unaffected by morphine treatment at PD7 and PD21. Some of the findings of this dissertation are straight forward, while others are difficult to interpret. Further emphasizing the difficulty of accurately interpreting data obtained from studies using young rodents.

Acknowledgements

This completed dissertation is a long time coming and there are many people who have been instrumental in helping me make it through to the other side.

I would like to thank my advisor Dr. Gordon Barr, who has taught me many things and who has given me the space to truly make this dissertation my own. I would also like to thank the remainder of my dissertation committee Dr. Jesus Angulo, Dr. Shirzad Jenab, Dr. Vanya Quiñones-Jenab, and Dr. Regina Sullivan for taking time out of their busy schedules to review my dissertation.

Many of the techniques used in this dissertation could not have been accomplished without the patient instruction from Dr. Christophe Wiedenmayer, Shaoning Wang, Jianxin Cheng, and Dr. Shirzad Jenab. In addition I would like to acknowledge Barbara Wolin, Sonja Acevedo, and the remainder of the Hunter College Animal Facility for the excellent care of my animals.

Past and present lab members are always near and dear to my heart. We may have gotten on each others' nerves from time-to-time, but we always supported each other. Many thanks go out to Jéan Willi, Dr. Hongbo Zhu, Dr. Estevan Ruis Limòn, Rebecca Smart (and Grace), and Dr. Arbi Nazarian. In addition, I will always be grateful to the assistance I received from Jose Guzman and Luincys Fernández, many of the experiments in this dissertation could not have been accomplished without them.

Of course I owe much support (technical and emotional) to my two girls…Sonja Stell and Dr. Amber Hodges. You always knew the story behind the story and we spent many days laughing our frustrations away.

I have saved the best for last. Thank you mom (Alicia) and Avin. There are many days that I would not have gotten through without your love, patience, support, and honesty. I am also eternally grateful to the support I received from my father (Winston), my stepmother (Emily), and my stepfather (Marty). I would also like to thank my little brother and sister (who are not so little anymore) for forgiving the fact that I do animal research. Finally, I would like to thank my grandparents (Alton Jones, Edith Jones, Rosarita McPhie, and the late Sydney McPhie) for loving me and gently reminding me that it is time for me to get this degree!

Thank you through it all...

Abbreviations

δ-	delta opioid receptor
κ-	kappa opioid receptor
μ-	mu opioid receptor
AC	adenylyl cyclase
ANOVA	analysis of variance
AP-1	activator protein-1
Aq	aqueduct
cAMP	cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
CaRE	Ca^{2+} response element
cGMP	cyclic quanosine monophophate
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
DA	dopamine
DAG	diacylglycerol
DH	dorsal horn (of spinal cord)
DMSO	dimethyl sulfoxide
DOR	delta opioid receptor
ED	embryonic day
ER	endoplasmic reticulum
FC	frontal cortex
GC	guanylyl cyclase
GD	gestational day
GRK	G protein-coupled receptor kinases
IEG	immediate early gene
IP_3	inositol 1,4,5-triphosphate
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.t.	intrathecal
KOR	kappa opioid receptor
LCGU	local cerebral glucose utilization
LC	locus coeruleus
-LIR	like immunoreactivity
MOR	mu opioid receptor
mRNA	messenger ribonucleic acid
NAcc	nucleus accumbens
NE	noradrenergic
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
OR	opioid receptor
PAG	periaqueductal gray area
PD	postnatal day
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol 4 monophosphate
PK	protein kinase
PKA	cAMP -dependent protein kinase
PKAri	PKA regulatory subunit 1
PKArii	PKA regulatory subunit 2
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PL-Ca-PK	phospholipid-sensitive Ca^{2+}-dependent protein kinase
POA	preoptic area
PP	protein phosphatase
PVG	periventricular gray substance
SC	spinal cord
SRE	serum response element
sVRP	slow ventral root potential
USV	ultrasonic vocalization
VTA	ventral tegmental area

Table of Contents

ABSTRACT / iv
ACKNOWLEDGEMENTS / vi
ABBREVIATIONS / vii
TABLE OF CONTENTS / viii
LIST OF TABLES / xi
LIST FIGURES / xii

PART ONE: THE OPIATES / 1

Chapter 1: An Introduction to Opiate Tolerance and Dependence / 2
• Background of Opiate Tolerance and Dependence / 2
• Opiate Tolerance and Dependence Defined / 4
• Opiate Tolerance and Dependence in the Adult Rodent / 7
• Opiate Tolerance and Dependence in the Infant Rodent / 8
• Summing up the General Similarities and Differences Across Ontogeny / 11
• Tables / 14

PART TWO: MECHANISMS UNDERLYING TOLERANCE AND DEPENDENCE / 16

Chapter 2: Communication Between Neurons / 17
• The Big Picture / 17
Activation of the cAMP/adenylyl cyclase pathway / 18
Activation of the IP_3-DAG Pathway / 18
A Brief Introduction to Protein Phosphatases / 19
General Cellular/Molecular Effects of Opiate Exposure / 20
Other Effects of Opiate Treatment: Further Downstream / 21
Communication Between the Two Pathways / 22
• Opioid Receptors / 23
• Protein Kinases / 26
Studies Using Non-Specific Protein Kinase Antagonists / 27
Studies Using Antagonists Specific to PKA / 27
Studies Using Antagonists Specific to PKC / 28
Changes in Levels of PKA / 28
Changes in Levels of PKC / 29
• Genes / 30
• Neuroanatomical Sites Corresponding with Opiate Tolerance and Dependence / 32
The Locus Coeruleus / 35
The Nucleus Accumbens / 36
The Periaqueductal Gray Area / 37

The Spinal Cord / 38
• Development of the Mechanisms Underlying Opiate Tolerance and Dependence / 38
• Mechanisms Underlying Opiate Tolerance and Dependence in the Young Rodent / 41
• Figures and Tables / 44

PART THREE: SPECIFIC AIMS / 51

Introduction / 52
• Specific Aim 1 / 54
• Specific Aim 2 / 54
• Specific Aim 3 /54

PART FOUR: GENERAL METHODS / 56

Materials/Methods / 57
• Subjects / 57
• Tattooing / 57
• Drug Preparation /58
• Treatment Schedules / 58
Acute / 58
Chronic / 58
Withdrawal Precipitation / 58
• Behavioral Assessment / 59
Withdrawal / 59
Tolerance / 59
• Table / 61

PART FIVE: WHERE TO START LOOKING IN THE YOUNG RAT – PART 1 OF YOUNG RAT EXPERIMENTS / 62

• Explanation / 63

Chapter 3: Receptor Mediation of Morphine Tolerance and Dependence / 65
• Introduction / 65
• Materials/Methods / 67
• Results / 68
• Discussion / 69
• Figures / 73

Chapter 4: Gene Expression During Morphine Tolerance and Dependence / 76
• Introduction / 76
• Materials/Methods / 79
• Results / 81
• Discussion / 82
• Figures / 89

PART SIX: THE PROTEIN KINASE AND OPIATE INTERACTION – PART 2 OF YOUNG RAT EXPERIMENTS / 94

• Explanation / 95

Chapter 5: The Modulation of Morphine Tolerance and Dependence by Protein Kinases (Behavioral Analysis) / 97
• Introduction / 97
• Materials/Methods / 99
• Results / 100
• Discussion / 101
• Figures and Tables / 106

Chapter 6: The Modulation of Protein Kinases by Morphine Treatment (Molecular Analysis) / 119
• Introduction / 119
• Materials/Methods / 122
• Results / 124
• Discussion / 126
• Figures and Tables / 133

PART SEVEN: GENERAL DISCUSSION / 142

• Summary of Overall Findings / 143
Receptor Mediation of Morphine Dependence / 144
Gene Expression During Morphine Dependence / 144
The Protein Kinase and Morphine Interaction / 144
•Discussion of Overall Findings / 145
• Future Directions / 147
• Table / 152

REFERENCES / 153

List of Tables

Table 1-1. Opiate Effects in Human Adults / 14

Table 1-2. Adult Rodent Opiate Withdrawal Signs / 15

Table 2-1. Opiate Effects on Different Brain Regions / 48

Table 2-2. Developmental of the Different Proteins / 50

Table GM-1. Withdrawal Behaviors During Ontogeny / 61

Table 5-1. Withdrawal Behavior Statistics (ANOVA, all grps) / 108

Table 5-2. Withdrawal Behavior Statistics (ANOVA, H-7+Ntx) / 111

Table 5-3. Withdrawal Behavior Mean Table (95% Conf.) / 114

Table 5-4. PD7 Tail-Flick Test Means / 117

Table 5-5. PD21 Tail-Flick Test Means / 118

Table 6-1. Acute Morphine Treatment Statistics / 135

Table 6-2. Chronic Morphine Treatment Statistics / 138

Table 6-3. Morphine Withdrawal Statistics / 141

Table GD-1. Similar/Different Mechanisms Throughout Development / 152

List of Figures

Figure 2-1. Acute Opiate Effects on the cAMP/AC Pathway / 44

Figure 2-2. Acute Opiate Effects on the IP_3-DAG Pathway / 45

Figure 2-3. Model of Opiate Effects on cAMP / 46

Figure 2-4. Brain Regions Effected by Opiates / 47

Figure 3-1. CTOP Effects on Withdrawal / 73

Figure 3-2. Naltrindole Effects on Withdrawal / 74

Figure 3-3. Nor-BNI Effects on Withdrawal / 75

Figure 4-1. Photomicrograph of the Periaqueductal Gray Area / 89

Figure 4-2. Nucleus Accumbens / 90

Figure 4-3. Periaqueductal Gray Area / 91

Figure 4-4. Dorsal Horn of the Spinal Cord / 92

Figure 4-5. Locus Coeruleus / 93

Figure. 5-1. PD7 Withdrawal Behaviors (ANOVA, all grps) / 106

Figure. 5-2. PD21 Withdrawal Behaviors (ANOVA, all grps) / 107

Figure 5-3. PD7 Withdrawal Behaviors (ANOVA, H-7+Ntx) / 109

Figure 5-4. PD21 Withdrawal Behaviors (ANOVA, H-7+Ntx) / 110

Figure 5-5. PD7 Withdrawal Behaviors (95% Conf.) / 112

Figure 5-6. PD21 Withdrawal Behaviors (95% Conf.) / 113

Figure 5-7. PD7 Tail-Flick Test / 115

Figure 5-8. PD21 Tail-Flick Test / 116

Figure 6-1. PD7 Acute Morphine Treatment / 133

Figure 6-2. PD21 Acute Morphine Treatment / 134

Figure 6-3. PD7 Chronic Morphine Treatment / 136

Figure 6-4. PD21 Chronic Morphine Treatment / 137

Figure 6-5. PD7 Morphine Withdrawal / 139

Figure 6-6. PD21 Morphine Withdrawal / 140

Part One: The Opiates

Chapter 1: An Introduction to Opiate Tolerance and Dependence

Background of Opiate Tolerance and Dependence

The term narcotic analgesic was originally used to exclusively describe opiates, but is now a common legal term used for any exogenous substance that has the potential for addiction (Hardman, Limbird et al. 2001), as well as the opiate family. The opiate family consists of opiates (eg. opium, morphine, and codeine), their derivatives and semisynthetic opioids (eg. heroin, nalorphine, and hydromorphine), synthetic opioids (eg. methadone, fentanyl, and LAAM.), opioid antagonists (eg. naloxone and naltrexone) and endogenous opioid peptides (β-endorphin, enkephalin, and dynorphin) (Grilly 1998; Julien 1998; Levine 2000; Hardman, Limbird et al. 2001). Each opiate has a different pharmacological effect, potency, intensity, duration of action, and oral effectiveness (Eddy, Halbach et al. 1965; Grilly 1998). However, they all have in common the ability to relieve pain and diarrhea; induce euphoria, drowsiness and mental clouding; and suppress cough (Collins 1985; Trujillo and Akil 1991; Levinthal 1996; Feldman, Meyer et al. 1997; Grilly 1998; Levine 2000; Hardman, Limbird et al. 2001). In addition, opiates exert an effect on thermoregulation, the neuroendocrine, cardiovascular, gastrointestinal, and immune systems (Feldman, Meyer et al. 1997; Hardman, Limbird et al. 2001).

Morphine, one of the 40 alkaloids contained within opium, is found endogenously in both invertebrates and vertebrates (Stefano, Goumon et al. 2000). Morphine was isolated from opium in 1803 and determined to be ten times more potent as an analgesic than smoked opium (Levinthal 1996; Feldman, Meyer et al. 1997; Julien 1998; Hardman, Limbird et al. 2001). Initially, morphine was added to tonics, where absorption through the gastrointestinal tract was slow and incomplete (Julien 1998). However, when the availability of hypodermic needles became more widespread to the general population, allowing it to enter directly into the bloodstream, doctors and laypeople discovered the effectiveness of morphine as an analgesic and euphoric agent (Feldman, Meyer et al. 1997).

One of the reasons opiates have a high abuse potential is that in addition to being unmatched in their ability to produce analgesia, they also produce powerful feelings of euphoria and sedation (Feldman, Meyer et al. 1997; Grilly 1998; Julien 1998). With no opiate is this more evident than with the morphine derivative heroin, which is 2-4 times more potent than morphine (Grilly 1998; Julien 1998). Heroin was initially introduced by the German company Bayer in 1898 as a more effective cough suppressant than codeine, but without the addictive properties of morphine (Levinthal 1996). Today it is common knowledge that heroin is in fact just as addictive, if not more addictive, than morphine.

As with morphine, the most common route of administration for heroin is directly into the bloodstream. Heroin is produced from the addition of two acetyl groups to a morphine molecule. The acetyl groups are fat-soluble and therefore are able to easily cross the blood-brain-barrier (Mestek, Hurley et al. 1995; Levinthal 1996; Feldman,

Meyer et al. 1997; Julien 1998). As a result, heroin reaches the brain at a much quicker rate than morphine and many abusers report feeling a sudden dramatic surge of pleasurable sensations, that serve as powerful reinforcement to continue using heroin (Julien 1998).

Major drawbacks from long-term heroin (and other opiates) use includes liver and kidney disease, scarred and/or collapsed veins, bacterial infections of the blood vessels and heart valves, and infection with hepatitis B, hepatitis C, HIV, or other blood-born viruses, as well as economic loss, property damage, and poor interpersonal relationships (Eddy, Halbach et al. 1965; Levine 2000; Policy 2001). As a result of the risks associated with injecting heroin, it became less popular as "the drug of choice" for a period of time. A new resurgence of heroin use began in the 1990's when the popularity of cocaine decreased, the purity of street heroin increased, and heroin became available in forms that could be smoked or snorted, instead of injected (Cooper, Bloom et al. 1996; Levinthal 1996; Office of Applied Studies 2001).

Opiate Tolerance and Dependence Defined.

An unfortunate, but real side effect of continuous (or even intermittent over a prolonged period of time) intake of any of the opiates is the development of tolerance and dependence, which can greatly limit its use in the clinic or increase the danger of its use illicitly (Collin and Cesselin 1991; Brodsky, Elliott et al. 1995). Both tolerance and dependence are the natural consequences of repeatedly introducing an exogenous substance into a biological system, and as a result, disrupting the homeostasis of that system (Feldman, Meyer et al. 1997; Fundytus and Coderre 1999; Hardman, Limbird et al. 2001; Bagley, Gerke et al. 2005; Dalton, Smith et al. 2005; McClung, Nestler et al.

2005). They are considered, by some, good examples of behavioral and cellular plasticity (Mayer, Mao et al. 1995; Trujillo 1995). Tolerance and dependence have typically been thought to be the result of the same adaptive pathways with both occurring at the same time (Collier 1965; Eddy, Halbach et al. 1965; Sharma, Klee et al. 1975; Traber, Gullis et al. 1975), however there is evidence that one can occur in the absence of the other (Schulz, Wuster et al. 1980; Rubini, Schulz et al. 1982; Schulz, Seidl et al. 1982; Andrade, Vandermaelen et al. 1983; Wüster, Schulz et al. 1985).

Tolerance is defined as a decreased responsiveness to a particular dose of a drug over time such that higher doses of the drug are needed to exert the same effect (Hanna 1960; Eddy, Halbach et al. 1965; Lotti, Lomax et al. 1966; Trujillo and Akil 1991; Chakrabarti, Law et al. 1995; Buzas, Rosenberger et al. 1996; Feldman, Meyer et al. 1997; Grilly 1998; Fundytus and Coderre 1999; Levine 2000; Hardman, Limbird et al. 2001). Pharmacologically it can be displayed as a rightward shift in a dose-response curve (Collin and Cesselin 1991; Trujillo and Akil 1991; Hardman, Limbird et al. 2001). Tolerance to the many effects of opiates develops at different time courses (Hardman, Limbird et al. 2001) or, in some cases, does not develop at all. For instance, tolerance develops to the analgesic, euphoric, respiratory depressant, thermoregulatory, and metabolic effects of opiates (Hanna 1960; Martin, Wikler et al. 1963; Trujillo and Akil 1991; Levinthal 1996; Levine 2000; Hardman, Limbird et al. 2001), but not to the effects of opiates on the gastrointestinal tract and pupil dilation (Levinthal 1996; Levine 2000).

Tolerance has been categorized in many different ways (Feldman, Meyer et al. 1997; Julien 1998; Levine 2000; Hardman, Limbird et al. 2001), all of which can be divided into three broad categories. 1) Behavioral (or learned) tolerance, where environmental

and drug associations are learned through experience (Trujillo and Akil 1991; Levine 2000; Hardman, Limbird et al. 2001). 2) Pharmacological tolerance, which includes pharmacodynamic and pharmacokinetic adaptations within the central nervous system (Trujillo and Akil 1991; Levine 2000; Hardman, Limbird et al. 2001). 3) Psychological tolerance, which is an adaptation to the feelings associated with the drug.

In addition to tolerance, long-term exposure to opiates results in dependence. The basic definition of dependence is the need for continued exposure to the drug either to experience its desirable effects or to avoid the associated unpleasant effects when it is no longer present (Eddy, Halbach et al. 1965; Buzas, Rosenberger et al. 1996; Kreek 1996; Grilly 1998). As with tolerance, dependence can be further divided into psychological and physical dependence, both of which have been demonstrated in animal models. Psychological dependence describes the emotional need to continue taking the drug to avoid the negative affect that can occur and it is regulated by the mesolimbic-dopamine system (Eddy, Halbach et al. 1965; Feldman, Meyer et al. 1997; Levine 2000).

Physical dependence describes the strong need to avoid extremely unpleasant, but not life-threatening, physical withdrawal behaviors that are manifested as behavioral changes, physiological changes, and neuronal changes (Eddy, Halbach et al. 1965; Trujillo and Akil 1991; Grilly 1998). These withdrawal behaviors can be relieved by the re-administration of the opiate and therefore are powerful reinforcers for the continuous use of the drug. (Eddy, Halbach et al. 1965). Dependence has also been studied because it is an excellent model of the way in which system adapts to change, since initial (or acute) exposure to opiates causes certain effects, while withdrawal from the same opiate causes effects that are opposite to those initially seen. (See Table 1-1).

Opiate Tolerance and Dependence in the Adult Rodent.

Numerous studies of opiate tolerance and dependence have been published over the last 50 years using multiple organisms including dogs, chickens, monkeys, and rodents (Martin, Wikler et al. 1963; Martin, Eades et al. 1976; Kuwahara and Sparber 1981; Gmerek, Dykstra et al. 1987; Sanchez-Blazquez, Rodriguez et al. 1996; Yu, Hao et al. 1997). They have all sought to understand the two phenomena in the adult organism in an attempt to more thoroughly understand them in human adults.

While it is well known that chronic treatment with an opiate results in tolerance (Hanna 1960; Rosenfeld and Burks 1977; Abdelhamid, Sultana et al. 1991; Yu, Hao et al. 1997) it is also possible to see tolerance with an acute dose of the opiate given systemically or intracerebrally (Lotti, Lomax et al. 1966; Rosenfeld and Burks 1977; Abdelhamid, Sultana et al. 1991). The degree to which tolerance develops is dependent on the dose of the opiate, the injection interval, and the potency of the opiate (Hanna 1960; Stevens 1994). In addition, activation of each of the opioid receptors can exert different effects on tolerance. For instance, the co-administration of an δ-opioid receptor antagonist with chronic morphine reduces the development of antinociceptive tolerance without effecting tolerance to the respiratory depressive effects of morphine (Abdelhamid, Sultana et al. 1991; Hepburn, Little et al. 1997). However, pre-treatment with a κ-opioid receptor antagonist followed by chronic morphine enhances the development of tolerance (Suzuki, Narita et al. 1992).

In addition to tolerance, animal models enabled researchers to study opiate dependence in the adult rodent, and as a result an array of physical withdrawal behaviors have been identified and described. (See Table 1-2). The degree to which each behavior appears is

effected by the dosage of the opiate agonist and/or antagonist, the frequency of opiate delivery, and the duration of opiate exposure (Bläsig, Herz et al. 1973). The behaviors are also effected by differential blockade of the opioid receptors such that the most robust display of withdrawal appears with blocked activity at the μ-opioid receptor and the weakest is produced by the blockade of the δ- and κ-opioid receptors (Cowan, Zhu et al. 1988; Maldonado, Negus et al. 1992b). Despite the details, the overall picture is that chronic morphine treatment causes dependence and the precipitated removal (or spontaneous) of the opiate results in an array of withdrawal behaviors (Martin, Wikler et al. 1963; Buckett 1964; Wooten, DiStefano et al. 1982; Geary and Wooten 1985; Yu, Hao et al. 1997; Le Guen, Gestreau et al. 2001). This withdrawal is also associated with increased regional cerebral glucose utilization (Wooten, DiStefano et al. 1982; Geary and Wooten 1985), increased levels of the Fos protein (Le Guen, Gestreau et al. 2001; Le Guen, Gestreau et al. 2003), reduced dopaminergic (DA) firing and burst rates (Diana, Pistis et al. 1995), and the appearance of conditioned place aversion (Spanagel, Almeida et al. 1994).

Opiate Tolerance and Dependence in the Infant Rodent

The vast majority of drugs ingested by pregnant women cross the placenta and, although not immediately, are able to enter the fetal circulation (Ward 1989). During early gestation brain concentrations of opiates are lower than that found in the mother, but later in gestation they become higher; possibly the result of a slower elimination rate and less developed blood-brain barrier (Peters 1975; Besunder, Reed et al. 1988; Ward 1989; Suresh and Anand 1998). In addition, higher concentrations of unbound morphine are found in the fetal umbilical cord and there is evidence that their rate of drug

absorption and excretion is lower than in the adult (Besunder, Reed et al. 1988; Reed and Besunder 1989).

Despite the unique pharmacokinetics and pharmacodynamics of the human fetus and newborn they are capable of expressing withdrawal symptoms, once the opiate has been removed from their CNS. The most serious outcome of neonatal opiate exposure is an increased rate of mortality (Finnegan 1985), although improved technology in the United States has greatly reduced its occurrence. Research using animal models has shown that the likely cause of these stillbirths and early deaths are in fact the result of in utero or postnatal withdrawal, and not merely the exposure to opiates (Kuwahara and Sparber 1981; Lichtblau and Sparber 1981; Sparber and Lichtblau 1983). The less life-threatening withdrawal behaviors seen within a few days of birth include hyperirritability, respiratory distress, fever, tremors, high-pitched crying, loose stools, and increased muscle tone (Zelson, Rubio et al. 1971; Rajegowda, Glass et al. 1972; Rothstein and Gould 1974; Finnegan 1985).

In addition, the weight of these infants at birth is often very low and they have smaller head circumferences (Rosen and Johnson 1982), possible results of poor nutrition and poly-drug use by the mother (Rothstein and Gould 1974; Finnegan 1985) during gestation. Long-term consequences of prenatal opiate exposure include altered neurological functioning (eg. problems with coordination and balance), decreased attention span, and lower IQ scores (Marcus, Hans et al. 1984; Kenner and D'Apolito 1997). Fortunately, many of these adverse effects can be minimized by the home environment in which the child is raised (Soepatmi 1994; Ornoy, Michailevskaya et al. 1996).

In the rodent literature there are also studies showing some of the long-term effects of opiate exposure during early gestational development. The treatment of dams before conception and/or throughout gestation with opiates results in adult offspring with a preference to self-administer methadone (Hovious and Peters 1985), an inhibited lordosis behavior (a female sexual posture), and reduced levels of estrodial and progesterone (Siddiqui, Haq et al. 1997). In addition, as with adult rodents, chronic exposure to opiates results in tolerance and dependence in the young rodent (Van Praag and Frenk 1991; Barr and Wang 1992d; Jones and Barr 1995; Windh, Little et al. 1995b).

Initially some studies were unable to detect analgesic tolerance to opiates until PD15 and 17 (Fanselow and Cramer 1988; Windh, Little et al. 1995b; Thornton, Wang et al. 1997). However, other studies were able to find tolerance as early as PD3-9 (Tempel, Habas et al. 1988; Van Praag and Frenk 1991; Barr and Wang 1992d). It is unclear why some studies were unable to detect tolerance during the first two weeks postnatally, but it is very likely a combination of the paradigms used and the operational definitions each laboratory established for successful removal of the target limb from the source of noxious stimuli. In addition, judging limb withdrawal with neonatal rodents can be difficult because they have a natural tendency to curl their limbs and tail into their bodies. Despite the early contradictory findings, it has now been well established that infant rodents experience opiate tolerance as early as the first week after birth (Tempel, Habas et al. 1988; Barr and Wang 1992d; Zhu and Barr 2003a), and therefore are appropriate animal models for infant tolerance in the clinical setting.

As with tolerance, opiate withdrawal (as an indicator of dependence) was initially not detected until PD17 by one study (Thornton, Wang et al. 1997) and as late as PD52 by

another study (Fanselow and Cramer 1988). Many other studies, however, have since found that pups as early as gestational day 20 display withdrawal behaviors (Jones and Barr 1995; Windh, Little et al. 1995b; Jones and Barr 2000; McPhie and Barr 2000; Maeda, Kishioka et al. 2002). The interesting idea that has been hypothesized and studied to explain the original discrepancies is that the withdrawal behaviors detected at different time points during early ontogeny are qualitatively different from those typically seen and described in the adult literature (Fanselow and Cramer 1988; Jones and Barr 1995; Windh, Little et al. 1995b; Jones and Barr 2000). This means that while a researcher may use piloerection and teeth chattering as an indication of withdrawal in the adult rodent, the behaviors they must look for during gestation are very likely less complex (or subtler) motor activities like body curls or mouth movements (Jones and Barr 2000). In addition to withdrawal behaviors, both conditioned place aversion and ultrasonic vocalizations in response to stimulation of the κ-opioid receptor and conditioned place preference after intra-ventral tegmental area injections of morphine are seen as early as PD4 (Barr and Rossi 1992c; Barr, Wang et al. 1994b).

Summing up the General Similarities and Differences Across Ontogeny.

As previously introduced, both opiate tolerance and dependence develop in the infant (Van Praag and Frenk 1991; Barr and Wang 1992d; Windh, Little et al. 1995b; Thornton, Wang et al. 1997) and adult (Tortella, Moreton et al. 1979; Christie, Williams et al. 1987; Adams and Wooten 1990; Maldonado, Negus et al. 1992b; Gold, Stinus et al. 1994) rodent. The μ-opioid receptor plays a critical, but not totally exclusive role in morphine induced withdrawal in the adult rodent (Kornblum, Hurlbut et al. 1987; Abdelhamid, Sultana et al. 1991; Christie, Williams et al. 1997) and likewise in the 7-day old rat the μ-

opioid receptor appears to play the primary role in observed withdrawal behaviors (McPhie and Barr 2000).

It is important, however, to understand that although there are similarities there are also significant differences in the way that infants and adults respond to long- and short-term exposure to opiates. For instance, there is evidence of the role of δ- and κ-opioid receptors in tolerance and dependence in the adult, although the limited data in the infant do not support their roles (McPhie and Barr 2000). These differences may be due to the staggered temporal development of the opioid receptor system and related systems (Duman, Tallman et al. 1988; Bernstein and Welch 1997; Jones and Barr 2001), in which the μ- and κ- ORs appear during gestation while the δ-OR does not appear until after birth. In addition, nitric oxide synthase inhibitors reduce withdrawal in both the infant and adult, but the NMDA receptors that play a role in opiate tolerance and dependence in the adult rodent, are not involved in morphine induced withdrawal and tolerance in the 7-day old rat (Zhu and Barr 2000; Zhu and Barr 2001a). These NMDA receptors do not block the behaviors until postnatal day 14 and 21 in the rat (Zhu and Barr 2000; Zhu and Barr 2001a).

There is also evidence that, at least acutely, μ-opioid receptor coupling to guanylyl nucleotide binding proteins is weaker in the infant than in the adult (Windh and Kuhn 1995a). Finally, the ability of PKC to translocate from the cytosol to the nucleus, the neuronal density in the cerebral cortex and cerebellum, the amount of dopamine binding, and the levels of PKC, cAMP, and Ca^{2+} channels are all altered by age (Battaini, Del Vesco et al. 1990; Hara, Onodera et al. 1992; Battaini, Elkabes et al. 1995).

These discrepancies between the adult and infant system make it clear that infants are not just "miniature" versions of adults. During early postnatal development they display extreme variability in the functioning of gastric emptying, the pancreas, levels of bound protein in the umbilical cord plasma, drug metabolism by the liver, and drug excretion by the kidney (Besunder, Reed et al. 1988; Reed and Besunder 1989). As a result, the infant system is distinct from adults and as such should be studied separately. The same adverse situation may have an effect on both infants and adults, for example alterations in cellular functioning as a result of exposure to opiates. However, because of the relative immaturity of the infant CNS their responses may not resemble the typical responses seen in adults, and thus may be interpreted differently. For instance, the blood-brain-barrier is less developed in the infant (Peters 1975) and therefore one would predict that more molecules of a given drug would interact with the CNS at a faster rate and exert a stronger effect. However, this may be neutralized by the fact that the receptors for that drug have not yet matured and as a result, the effect of the drug is less potent in the infant than in the mature adult. For these reasons, the overall purpose of this dissertation is to provide further evidence of the similarities and/or differences between the infant and adult CNS during opiate tolerance and dependence.

Opiate Effects in Human Adults

Acute action	Withdrawal sign
Analgesia	Pain & irritability
Respiratory depression	Hyperventilation
Euphoria	Dysphoria & depression
Relaxation and sleep	Restlessness & insomnia
Tranquilization	Fearfulness & hostility
Decreased blood pressure	Increased blood pressure
Constipation	Diarrhea
Pupullary constriction	Pupullary dilation
Hypothermia	Hyperthermia
Drying of secretions	Lacrimation, runny nose
Reduced sex drive	Spontaneous ejaculation
Peripheral vasodilation flushed and warm skin	Chilliness & “gooseflesh”

Table 1-1. Shows the acute effects of opiate treatment as they compare to the effects seen after chronic opiate treatment followed by precipitated withdrawal. Table taken Feldman, 1997.

Adult Rodent Opiate Withdrawal Signs

Withdrawal signs	References
Diarrhea	(Buckett 1964; Bläsig, Herz et al. 1973; Maldonado, Feger et al. 1990; Punch, Self et al. 1997; Schulteis, Heyser et al. 1997)
Ptosis	(Buckett 1964; Bläsig, Herz et al. 1973; Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b; Punch, Self et al. 1997; Schulteis, Heyser et al. 1997)
Piloerection	(Martin, Eades et al. 1976; Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b)
Rearing	(Maldonado, Negus et al. 1992b; Punch, Self et al. 1997)
Wet-dog shakes	(Buckett 1964; Bläsig, Herz et al. 1973; Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b)
Tremor	(Martin, Eades et al. 1976)
Spontaneous ejaculation	(Bläsig, Herz et al. 1973; Punch, Self et al. 1997; Schulteis, Heyser et al. 1997)
Head tossing	(Martin, Eades et al. 1976)
Grooming	(Punch, Self et al. 1997)
Jumping	(Bläsig, Herz et al. 1973; Maldonado, Feger et al. 1990; Punch, Self et al. 1997; Schulteis, Heyser et al. 1997)
Mastication	(Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b)
Irritability	(Punch, Self et al. 1997; Schulteis, Heyser et al. 1997)

Table 1-2. A partial listing of the various classic withdrawal signs identified in the adult rodent, chronically treated with an opiate and then given an acute injection of an opiate antagonist.

Part Two: Mechanisms Underlying Tolerance and Dependence

Chapter 2
Communication Between Neurons

The Big Picture.

The binding of an extracellular opiate to its G-protein coupled receptor (μ-. κ-, and δ-OR) is the starting point of a complex sequence of events that define the long- and short lasting physiological and behavioral effects of opiates (Nestler 2004; Dalton, Smith et al. 2005; McClung, Nestler et al. 2005). These effects end with the transmission of information to numerous receiving neurons throughout the brain and spinal cord. In between there exist a cascade of changes in molecules downstream of the receptor, that transduce the action of receptor binding into biological responses (Childers 1991; Liu, Zhang et al. 2002) that society recognizes as opiate addiction (or dependence). Two of the major cellular transduction pathways that are well established to be directly involved in the cellular and molecular effects of opiates on the CNS are the cAMP/adenylyl cyclase and IP_3-DAG pathways (Chartoff, Papadopoulou et al. 2003; Bie and Pan 2005; Chakrabarti, Regec et al. 2005). A recent study was able to successfully co-immunoprecipitate Gβ, Giα, PKCγ, and AC from radiolabled Chinese Hamster Ovary cells transfected with μ-ORs (Chakrabarti, Regec et al. 2005). In addition, they found that levels of each of the protein kinases were increased with chronic morphine treatment and abolished by the administration of a PKCγ antagonist (Chakrabarti, Regec et al. 2005).

Activation of the cAMP/adenylyl cyclase pathway. The cascade of events within the cAMP/adenylyl cyclase (AC) pathway begins with the activation of a G-protein, whose α-subunit separates from its βγ-subunits and phosphorylates AC (Busquets, Escriba et al. 1995; Satoh and Minami 1995; Ventayol, Busquets et al. 1997; Cerezo, Laorden et al. 2002; Chakrabarti, Liu et al. 2003b). AC is then able to increase production of the second messenger cAMP, which binds to the regulatory (R) subunits of the protein kinase cAMP-dependent protein kinase (PKA), causing them to separate from their catalytic (C) subunits (Heller, Vigil et al. 2004; Kim, Xuong et al. 2005). The R subunits phosphorylate substrate proteins in the immediate cytoplasmic area, while the C subunits are able to diffuse into the nucleus and regulate gene transcription (Tasken and Aandahl 2004; Dalton, Smith et al. 2005) (See Figure 2-1).

Activation of the IP_3-DAG pathway. As with the cAMP/AC system, the cascade of events that are part of the IP_3-DAG system also begin with the activation of a G-protein. The G-protein then activates the primary effector phospholipase C (PLC) (Miyamae, Fukushima et al. 1993; Ueda, Miyamae et al. 1995; Ventayol, Busquets et al. 1997; Cerezo, Laorden et al. 2002; Chakrabarti, Liu et al. 2003b; Smith, Smith et al. 2004), which initiates the hydrolysis of phosphatidylinositol 4-monophosphate (PIP_2) to yield the two second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Narita, Mizoguchi et al. 2001a). IP_3 travels to the cytoplasm and releases Ca^{2+} from endoplasmic reticulum (ER) stores, by binding to its IP_3-gated channels (Smart and Lambert 1996a; Narita, Mizoguchi et al. 2001a). The increased levels of cytosolic Ca^{2+} cause Ca^{2+}-dependent protein kinase C (PKC) to translocate to the cell membrane (Alberts, Bray et al. 1994). DAG which has remained in the cell membrane,

phosphorylates the regulatory subunit of PKC and activates it (Narita, Mizoguchi et al. 2001a; Chakrabarti, Liu et al. 2003b). The phosphorylated PKC then travels back into the cytoplasm where it is involved in the phosphorylation of multiple proteins (Narita, Feng et al. 1994b; Lee, Hahm et al. 2004), including the modulation of μ-OR desensitization (Mestek, Hurley et al. 1995). (See Figure 2-2).

A brief introduction to protein phosphatases. Phosphorylation by protein kinases is a major step in neuronal signalling (Moncada, Cendan et al. 2003). However, for normal neuronal functioning the system must be balanced and therefore there exist a mechanism for dephosphorylation (Moncada, Cendan et al. 2003). The dephosphorylation, or removal of the phosphate group that is added by a protein kinase, is done by protein phosphatases (PPs) on the serine or threonine residues of the target proteins (Moncada, Cendan et al. 2003). There are numerous PPs within the CNS, but at this time the greatest amount of information is known about type1 protein phosphatases (PP1), and types 2A, 2B, and 2C (PP2A, PP2B, PP2C, respectively) (Muranyi, Gergely et al. 1997; Moncada, Cendan et al. 2003) protein phosphatase.

Of particular interest is the fact that protein phosphatases interact with the opiate system *in vitro* (Muranyi, Gergely et al. 1997) and *in vivo* (Bernstein and Welch 1998; Liu, Zhang et al. 2002; Moncada, Cendan et al. 2003). Bersnstein (Bernstein and Welch 1998) hypothesized that it is likely that in addition to PP inactivation blocking the occurrence of dephosphorylation, it may also enhance the ability of protein kinases to phosphorylate proteins. At the same time, the protein kinase PKC has been shown to have the ability to prevent PP1 from dephosphorylating substrate proteins (Liu, Zhang et al. 2002). Therefore, although they are not a focus of the current dissertation, protein

phosphatases are an integral part of protein phosphorylation/dephosphorylation and the subsequent behavioral and cellular effects of chronic opiate exposure (Moncada, Cendan et al. 2003). Therefore, their role in opiate tolerance and dependence will be discussed further in the General Discussion section of this dissertation.

General cellular/molecular effects of opiate exposure. Although there are contradictory findings (For example, nanomolar doses of opiate agonists enhance the action potentials seen in mouse dorsal root ganglion neurons of the SC, while micromolar doses decrease them (Collin and Cesselin 1991)), the overall picture theorized and supported is that acute administration of opiates reduces the activity and/or levels of the components of the cAMP/AC and IP_3-DAG pathways (Sharma, Klee et al. 1975; Duman, Tallman et al. 1988; Childers 1991; Collin and Cesselin 1991; Bernstein and Welch 1997; Liu and Anand 2001). Chronic exposure to the opiates then results in tolerance to their effects as the activity and/or levels of the proteins increase back to baseline levels (Sharma, Klee et al. 1975; Duman, Tallman et al. 1988; Childers 1991; Lane-Ladd, Pineda et al. 1997; Nestler and Aghajanian 1997). Finally, evidence of the development of dependence becomes apparent by an increase in activity and/or levels, dramatically beyond baseline, after the administration of an opiate antagonist (Sharma, Klee et al. 1975; Childers 1991; Beckmann, Matsumoto et al. 1995; Aley and Levine 1997b; Blendy and Maldonado 1998). (See Figure 2-3).

One explanation for the change between chronic and acute opiate administration is that ORs undergo a conformational change (Suresh and Anand 1998; Liu and Anand 2001). Such that initially they are coupled to inhibitory G-proteins (G_i) and at some point, after prolonged exposure to opiates, they switch their coupling to stimulatory G-proteins (G_s)

(Suresh and Anand 1998; Liu and Anand 2001); although this explanation requires further exploration.

Other effects of opiate treatment: Further downstream. The activation of the cAMP/AC and IP_3-DAG pathways also involves direct modulation of ion channels for K^+ (Bernstein and Welch 1995; Nestler and Aghajanian 1997; Liu and Anand 2001), Na^+ (Childers 1991; Nestler and Aghajanian 1997), and Ca^{2+} (Di Chiara and North 1992; Bernstein and Welch 1995; Suresh and Anand 1998; Smith, Dombrowski et al. 1999a) by G-proteins or the indirect modulation by PKA or PKC induced phosphorylation. The efflux of K^+ and influx of Na^+ then results in changes in neuronal polarization. Upon its entry into the cytoplasm, through channels or release from the ER, Ca^{2+} modulates the release of the endogenous neurotransmitter and binds to numerous proteins including calmodulin, thereby activating Ca^{2+}/calmodulin-dependent PK (CaMKII) (Alberts, Bray et al. 1994; Mestek, Hurley et al. 1995; Liu and Anand 2001) and G protein-coupled receptor kinases (GRK) (Penn, Pronin et al. 2000). CaMKII has the ability to phosphorylate μ-ORs (which can desensitize the activation of the inward K^+ current by the receptor (Mestek, Hurley et al. 1995)) and ntiric oxide synthase, which converts L-arginine into citrulline and forms the diffusible gas nitric oxide (NO). NO is then able to diffuse into neighboring neurons and activate guanylyl cyclase (GC) (Pasternak, Kolesnikov et al. 1995; Bhargava and Cao 1997), which increases production of the second messenger cGMP and is involved in the continued release of the neurotransmitter glutamate. Glutamate binds to NMDA (which are often co-localized with ORs on the postsynaptic cell) and Group 1 metabotropic glutamate receptors (mGlu) to regulate long-term opiate effects, such that blocking their activity at these receptors attenuates the

expression/development of opiate tolerance and dependence (Trujillo and Akil 1991; Mayer, Mao et al. 1995; Martin, Ahmed et al. 1999a; Smith, Smith et al. 2004).

In addition to the multiple cytoplasmic events, many of the protein kinases are able to travel into the nucleus and directly regulate gene transcription. For instance, both PKA and CaMKII phosphorylate cAMP response element binding protein (CREB) (Nestler 1992; Blendy and Maldonado 1998), which binds to the CaRE and CRE regulatory subunits of the immediate early gene (IEG) *c-fos*, genes for endogenous opioids like dynorphin (Sheng and Greenberg 1990; Feldman, Meyer et al. 1997; Blendy and Maldonado 1998; Nestler 2004), genes for other neuropeptides, signalling proteins, and other transcription factors (Nestler 2001). In addition, PKC phosphorylates the transcription factor serum response factor (SRF), which binds to the serum response element (SRE) regulatory subunit of the *c-fos* gene. The protein product of *c-fos*, Fos, forms heterotrimer complexes with other IEG products (eg. Jun) and regulates the transcription of late response genes, including genes for the opioid peptides preproenkephalin, prodynorphin, and proopiomelenocortin, by binding to their AP-1 regulatory subunit (Chang, Squinto et al. 1988; Beckmann, Matsumoto et al. 1995; Feldman, Meyer et al. 1997). (See Figures 2-1 and 2-2). In this way, an intracellular network of interacting systems is able to transduce the binding of an opiate to its receptor into a complex behavior, with possible long-term consequences.

Communication between the two pathways. It is also important to note, that although the cAMP/AC and IP_3-DAG signal transduction systems tend to be mentioned as if they are activated in a parallel manner there is evidence that cross-communication between them (Busquets, Escriba et al. 1995; Garcia-Sevilla, Ventayol et al. 1997; Ventayol, Busquets

et al. 1997) exist. For instance, *in vitro* studies have shown that PKC directly mediates the phosphorylation of the catalytic subunit of adenylyl cyclase (Yoshimasa, Sibley et al. 1987; Zhou, Curran et al. 1994), increases levels of cAMP (Zhou, Curran et al. 1994), and phosphorylates the α-subunit of the G_i-protein, suppressing its ability to inhibit adenylyl cyclase activity (Katada, Gilman et al. 1985). Both subunits of PKA and PKC have shown the ability to phosphorylate the Gβ-protein (although not the Gγ subunit) (Chakrabarti and Gintzler 2003a). In addition, in a strain of yeast with a mutation which causes it to produce lowered levels of cAMP, the activity levels of phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PIP), the two enzymes that catalyze the step from PI to PIP_2 in order to produce DAG and IP_3, are also reduced (Kato, Uno et al. 1989). There is also evidence of cross-communication from *in vivo* studies where the down-regulation of PKCαβ immunoreactivity in the frontal cortex of heroin addicts and morphine treated rats (Busquets, Escriba et al. 1995) is negatively correlated with the up-regulation of $G\alpha_{i1/2}$ -protein in the frontal cortex of the same populations (Escriba, Sastre et al. 1994). Based on the above *in vivo* findings Narita et al (Narita, Makimura et al. 1994c; Tokuyama, Feng et al. 1995a) proposed that the up-regulation of PKA activity in the LC by chronic opiate treatment may therefore lead to a comparable increase in PKC activity in the pons/medulla area, which consist of the LC, resulting in a more robust signal.

Opioid Receptors

Opiates exert their short-term and long-term physiological and behavioral effects through cell surface opioid receptors (ORs), which are located throughout the brain and SC of mammals (Tempel and Zukin 1987; Garcia, Brown et al. 1995; Mansour, Fox et al.

1995; Margolis, Hjelmstad et al. 2003). Although opiates have been known of and used for thousands of years, it was not until the early 1970's, with improved binding studies using radiolabled opioid ligands (Satoh and Minami 1995), that data was published with evidence supporting the speculation that receptors explicitly for opiates exist (Pert and Snyder 1973; Simon, Hiller et al. 1973). Later studies showed that there were three major opioid receptor types: the μ-OR (so named because it has a high affinity for morphine), the κ-OR (because it has a high affinity for the opiate ketocyclazocine), and the δ-OR (because it is concentrated within the mouse vans deferens) (Martin, Eades et al. 1976; Lord, Waterfield et al. 1977).

All three receptor types are members of a superfamily of G-protein coupled receptors, which possess seven-membrane spanning domains (Collin and Cesselin 1991; Satoh and Minami 1995; Julien 1998). Initial activation of any of the ORs causes activation of G_s-, G_i-, or G_o-coupled proteins (Parolaro, Rubino et al. 1993; Escriba, Sastre et al. 1994; Makimura, Narita et al. 1995; Garcia-Sevilla, Ventayol et al. 1997), located in the cell plasma membrane, resulting in down-regulation of signal transduction by modulation of K^+, Ca^{2+}, and Na^+ channels (Blume, Lichtshtein et al. 1979; Hescheler, Rosenthal et al. 1987; North, Williams et al. 1987; Rasmussen, Beitner-Johnson et al. 1990; Childers 1991; Cruciani, Dvorkin et al. 1993; Chen and Yu 1994; Mestek, Hurley et al. 1995; Han, Cho et al. 1999; Wang and Sadee 2000; Tan, Groszer et al. 2003; Chakrabarti, Liu et al. 2003b). When researchers initially began to look at the molecular mechanisms underlying tolerance and dependence, they assumed that changes in receptor numbers, function, and/or their subsequent uncoupling from the G-proteins are what underlie opiate tolerance and dependence (Collin and Cesselin 1991). However, the findings were

contradictory such that some found an up-regulation of opioid receptors after chronic opiate exposure (Rothman, Danks et al. 1986; Danks, Tortella et al. 1988; Brady, Herkenham et al. 1989; Yoburn, Billings et al. 1993), some found a down-regulation (Law, Hom et al. 1983; Werling, McMahon et al. 1989; Bhargava and Gulati 1990; Chakrabarti, Law et al. 1995; Sternini, Spann et al. 1996; Bernstein and Welch 1998; Kramer and Simon 1999b), and others found no changes (Bhargava and Gulati 1990; Brodsky, Elliott et al. 1995; Buzas, Rosenberger et al. 1996; Sternini, Spann et al. 1996; Castelli, Melis et al. 1997) in levels. In addition, tolerance and dependence last much longer than any changes in OR density and therefore the current belief is that the downstream events that occur as a result of OR stimulation more readily explain the long-term adaptive changes that define tolerance and dependence (Terwilliger, Beitner-Johnson et al. 1991; Liu and Anand 2001; Belanger, Ma et al. 2002).

However, the powerful role that ORs play in the development and manifestation of tolerance and dependence in the rodent model cannot be denied. This is especially apparent with pharmacological studies that have focused on the behavioral consequences of blocking activity at each receptor (Cowan, Zhu et al. 1988; Maldonado, Negus et al. 1992b). For instance, chronic activation of the μ-OR followed by treatment with an opiate antagonist results in a greater number of physical withdrawal behaviors, that are also qualitatively different (Gmerek, Dykstra et al. 1987), than those seen from activation and blockade of the κ- and δ-ORs (Young and Khazan 1985; Cowan, Zhu et al. 1988; Le Guen, Gestreau et al. 2003). When all receptor types are acutely activated analgesia with the formalin test develops, but only the μ-OR produces significant analgesia in the hot-plate and tail-flick tests (Maldonado, Feger et al. 1990). Within the NAcc, activation of

the μ-OR increases DAergic firing (within the mesolimbic dopamine system) while κ-OR activation decreases basal DAergic firing (Spanagel, Almeida et al. 1994), an effect which may be involved in the aversive component of opiate withdrawal (Pothos, Rada et al. 1991; Rossetti, Hmaidan et al. 1992). It appears that although each receptor regulates tolerance and dependence, the degree to which each one is involved differs, and furthermore, there is evidence that the μ-, κ-, and δ-ORs interact and regulate each others' activity (Suzuki, Narita et al. 1992; Maldonado, Negus et al. 1992b).

With regard to infants, there is evidence that pre- and postnatal treatment with morphine down-regulates μ-ORs (Tempel, Habas et al. 1988) and that ultrasonic vocalizations (USVs) are decreased by μ- and δ-OR agonists and increased by κ-OR agonists (Carden, Barr et al. 1991). However, two broad questions have remained relatively unexplored. What are the roles that each receptor plays in opiate tolerance and dependence at the behavioral level? How do the receptors modulate tolerance and dependence at the molecular level? Therefore, chapter 3 of this dissertation explores the role that each receptor plays in opiate dependence at the behavioral level in the young rat.

Protein Kinases

When stimulated by a second messenger molecule a protein kinase (PK) has the ability to phosphorylate a target protein, a crucial step in the signal transduction pathway that is essential for long-term cellular adaptation (Chen and Yu 1994; Liu and Anand 2001) to opiate exposure. A vast amount of research has focused on the regulation of opiates on the two protein kinases PKA and PKC and the second messengers that directly stimulate them.

Changes in the levels of the second messenger cAMP are difficult to determine directly, so many studies make the assumption that changes in activity level of AC are indicative of changes in levels of cAMP (Duman, Tallman et al. 1988). As expected acute morphine reduces AC activity in multiple brain regions (Duman, Tallman et al. 1988; Fleming, Ponjee et al. 1992), while chronic morphine increases phosphorylation by AC (Sharma, Klee et al. 1975; Duman, Tallman et al. 1988; Chakrabarti, Wang et al. 1998c), an effect that can be attenuated by a PKC antagonist (Chakrabarti, Wang et al. 1998c). As a result of the increased AC activity, levels of cAMP also increased, which can be further increased by the addition of an opiate antagonist (Sharma, Klee et al. 1975).

Studies using non-specific protein kinase antagonists. The strongest findings on the regulation of opiate tolerance and dependence by protein kinases are from studies that use non-specific antagonists. The most commonly used antagonists are H-7 and its analog H-8 (both inhibit PKA, PKC, and PKG). Tolerance to morphine antinociception is blocked by the administration of H-7 (Narita, Feng et al. 1994a; Bilsky, Bernstein et al. 1996), and both H-7 and H-8 attenuate behavioral (Mao, Price et al. 1995; Tokuyama, Feng et al. 1995a; Tokuyama, Ho et al. 2000) and affective signs of opiate withdrawal.

Studies using antagonists specific to PKA. PKA has an important regulatory role in opiate tolerance and dependence and its ability to exert an influence is based on the length of exposure to the opiate. After chronic exposure to morphine, the acute administration of PKA specific antagonists causes a reversal in tolerance (Bernstein and Welch 1997; Dalton, Smith et al. 2005) and an attenuation of withdrawal behaviors (Punch, Self et al. 1997). However, when rodents are given an acute dose of morphine followed by an acute dose of a PKA antagonist, they show no significant effect on their

development of tolerance (Narita, Mizoguchi et al. 1995; Inoue and Ueda 2000). Therefore it appears, for reasons that are not yet known, that PKA becomes important to the development of tolerance (and perhaps dependence) only after chronic exposure to opiates.

Studies using antagonists specific to PKC. Opiate tolerance and dependence is also modulated by PKC. The acute tolerance that develops is reversed by a specific PKC antagonist or blocked by pretreatment with the same PKC antagonist (Inoue and Ueda 2000). When rodents are continuously exposed to morphine spinally (Granados-Soto, Kalcheva et al. 2000) and supraspinally (Fundytus and Coderre 1996), a number of structurally unrelated PKC antagonists reverse tolerance and attenuate the development of dependence. Unlike PKA, PKC's ability to exert an influence on the development of these two phenomena is not based on the duration of exposure to the opiate. Withdrawal signs, whether they are the result of acute or chronic exposure to opiates, are decreased by PKC antagonists.

Another area of interest is the way in which exposure to morphine effects the intracellular levels of protein kinases. Various techniques have been employed to quantify the changes in the levels of PKA and PKC.

Changes in the levels of PKA. Within the locus coeruleus acute exposure to morphine causes a decrease in the levels of PKA (Duman, Tallman et al. 1988), whereas chronic exposure to opiates results in increases in the levels of PKA (Nestler and Tallman 1988; Nestler, Alreja et al. 1994) in the same brain region. In addition, the chronic co-injection of an opiate antagonist with morphine blocks the effects that chronic morphine exposure has on the levels of PKA (Nestler and Tallman 1988). Finally, levels of the PKA-Cα

subunit are significantly increased during morphine withdrawal in the adult rat (Benavides, Laorden et al. 2003).

Changes in the levels of PKC. In the pons/medulla region of rats chronically receiving opiates, there is an increase in the activity of PKC located within the cytosol (Narita, Makimura et al. 1994c; Tokuyama, Feng et al. 1995a), as well as an increase in the translocation of PKC from the cytosol to the membrane within the spinal cord (Mayer, Mao et al. 1995). In addition, repeated intrathecal (directly into the spinal cord) administration of morphine increases PKCγ and PKCα immunoreactivity (an effect that is reversed by an acute dose of a PKC antagonist) (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000) and increases PKC phosphorylation within the spinal cord (Granados-Soto, Kalcheva et al. 2000). Finally chronic opioid treatment increases levels of PKCγ in cell cultures (Chakrabarti, Regec et al. 2005) and PKCα-IR of dorsal root ganglion neurons (Belanger, Ma et al. 2002). However, other studies have found a significant decrease in the immunoreactivity of the PKCα and PKCβ isoforms in the frontal cortex of both humans who chronically used heroin and rats who were chronically exposed to morphine (Busquets, Escriba et al. 1995; Garcia-Sevilla, Ventayol et al. 1997). The discrepancies between the changes in levels of PKC may be the result of the different anatomical regions that were studied and/or the different isoforms measured. PKCγ is found in high concentrations in the spinal cord (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000), however only about 5% of those neurons contain both PKCγ and the μ-opioid receptor (Granados-Soto, Kalcheva et al. 2000). Which means that within the spinal cord and throughout the CNS other PKC isoforms play a role

in the regulation of opiate tolerance and dependence and studying just one or two isoforms may not present the entire picture.

All components of cellular signal transduction systems are important for the long-term and short-term changes caused from stimulation by the opiates, but PKs are particularly important because of their role in phosphorylation of certain proteins like genes, secondary effectors, and opioid receptors. Chapters 5 and 6 of this dissertation describe two experiments that were designed to assess the interaction between PKA/PKC and opiate tolerance and dependence in the developing rat model.

Genes

With long-term exposure to opiates there are permanent changes that the target neuron undergoes, such as changes in dendritic branching, changes in the production rate of proteins and/or changes in the expression of genes (Feldman, Meyer et al. 1997; Nestler 2001). This neuronal plasticity is believed to be mediated (in part, if not entirely) by gene expression (Guitart, Thompson et al. 1992; Blendy and Maldonado 1998) and is manifested as changes in the behaviors associated with tolerance and dependence. A crucial step in the regulation of gene expression is the binding of a transcription factor to the regulatory domain of the target gene (Chartoff, Papadopoulou et al. 2003). The manner in which the transcription factor cAMP response element binding protein (CREB) and the gene *c-fos* regulate and are regulated by chronic opiate exposure, has been thoroughly investigated.

One mechanism through which opiates exert their effect on CREB is through its phosphorylation by cAMP-activated PKA, which then allows it to regulate gene expression by binding to the regulatory domain of a gene. In the LC, CREB

phosphorylation is decreased by acute *in vivo* morphine treatment, brought back up to control levels by chronic morphine treatment, and increased beyond control levels by acute withdrawal (Guitart, Thompson et al. 1992; Chartoff, Papadopoulou et al. 2003); effects that are consistent with other findings on opiates and the cAMP/AC signal transduction system. However, in the NAcc CREB immunoreactivity is down-regulated with chronic morphine treatment, an effect (artificially recreated by the infusion of a CREB antisense oligonucleotide) that results in reduced immunoreactivity of the α- and catalytic subunits of the G_i-protein and PKA, respectively (Widnell, Self et al. 1996). The different results with chronic morphine treatment are very likely the result of the different brain regions studied and the different techniques used. Finally, while CREBα and CREBΔ mutant mice show a dramatic decrease in withdrawal signs and less robust tolerance to morphine analgesia, they show no effect in levels of *c-fos* and adenylyl cyclase activity during withdrawal (Maldonado, Blendy et al. 1996; Blendy and Maldonado 1998) or a preference for the morphine-paired side of a chamber (Walters and Blendy 2001). It is possible that CREBβ (the third transcriptionally active CREB isoform) has the ability to compensate for the loss of CREBα and CREBΔ or that *c-fos* and adenylyl cyclase are regulated by additional transcription factor systems (Maldonado, Blendy et al. 1996) or other members of the CREB family (Blendy and Maldonado 1998).

In addition to CREB, there is an immense amount of data on the gene *c-fos,* and its protein product Fos (which itself is a transcription factor that regulates the synthesis of other genes) (Beckmann, Matsumoto et al. 1995; Feldman, Meyer et al. 1997), and their regulation by the opiate system. In the forebrain, acute treatment with morphine

increases levels of *c-fos* mRNA (Chang, Squinto et al. 1988) and immunoreactivity (Frankel, Harlan et al. 1999a), as well as Fos-like immunoreactivity (LIR) (Chang, Squinto et al. 1988; Garcia, Brown et al. 1995). As expected, acute withdrawal from morphine causes significant increases in levels of Fos-LIR in the forebrain (Beckmann, Matsumoto et al. 1995; Gracy, Dankiewicz et al. 2001; Veinante, Stoeckel et al. 2003), SC (Rohde, Detweiler et al. 1996), and brainstem (Hayward, Duman et al. 1990; Chieng, Keay et al. 1995b). Opiate withdrawal also causes a dramatic increase in *c-fos* mRNA expression in many brain regions, including some that are rich in opioid receptors, as well as the spinal cord (Hayward, Duman et al. 1990; Beckmann, Matsumoto et al. 1995; Georges, Stinus et al. 2000). The increased levels of Fos protein during opiate withdrawal can be attenuated by chronic co-treatment of a PKA antagonist with morphine (Benavides, Laorden et al. 2003). In an attempt to better understand gene regulation in the young rodent by the opiate system, chapter 4 of this dissertation describes the effects of opiate withdrawal on levels of Fos-LIR within different brain regions, in the 7-day old rat.

Neuroanatomical Sites Corresponding with Opiate Tolerance and Dependence

Understanding the intracellular signal transduction systems involved in opiate tolerance and dependence also involves knowledge about the neuroanatomical sites within which they are located. When different sites are taken into account, each with its own distinct neuronal population, it quickly becomes apparent that, for instance, up-regulation in the LC does not guarantee up-regulation in the NAcc or PAG.

All drugs of abuse, including opiates, alcohol, nicotine, and cocaine are believed to have one final neuroanatomical pathway in common. This pathway is commonly

referred to as the mesolimbic dopamine (DA) system and it begins in the midbrain ventral tegmental area (VTA) and sends projections to rostral brain structures that include the substantia nigra, nucleus accumbens (NAcc), and the frontal cortex. Therefore if, for instance, an opiate is injected into the VTA, it inhibits local GABAergic neurons, which normally inhibit the activity of the postsynaptic DAergic neurons on which they synapse. As a result, DA is released within the mesolimbic system, producing the positive affect (or positive reinforcement) that is normally associated with fighting, feeding, fleeing, and copulating (behaviors essential for the survival of the organism) (Leshner 1996).

In addition to the psychological reinforcement of the opiate, information must be passed on to motor areas that can execute the appropriate behavior(s) needed to continue (or initiate) the intake of the drug and produce the feelings of pleasure (Shippenberg and Elmer 1998). As a result each time an addictive drug is taken by a human or laboratory animal, multiple interacting circuits are stimulated. They can be broadly grouped into three categories: Autonomic, motivational, and behavioral. However there is a great deal of overlapping among these categories.

A review of the literature has produced twelve primary neuroanatomical locations that have been shown to be consistently involved in opiate reward, as well as opiate tolerance and dependence (See Figure 2-4 and Table 2-1). They include (but are not limited to) the amygdala, frontal cortex, hippocampus, hypothalamus, locus coeruleus (LC), NAcc, N. raphe magnus, periaqueductal gray (PAG), spinal cord (SC), substantia nigra, thalamus, and VTA. Normally each one has its own function within the CNS to aid in the survival of the organism, however when an opiate is introduced to the CNS all regions play a collective role in the appearance of tolerance and dependence. Each region contains a

significant population of μ-, κ-, and δ-opioid receptors (although the proportion of each is different) and/or receives projections from opioid peptide containing neurons (Calvino, Lagowska et al. 1979; Wooten, DiStefano et al. 1982; Koob and Bloom 1988; Przewlocka, Turchan et al. 1996; Van Bockstaele, Colago et al. 1996; Chieng and Williams 1998; Shippenberg and Elmer 1998; Bear, Connors et al. 2001).

Determining the exact role that individual brain regions play is nearly impossible because each brain region receives thousands of connections from multiple sites and sends thousands of projections to multiple sites. For instance, the LC sends noradrenergic (NE) projections to basically every region of the CNS and therefore has been shown to play a role in arousal, attention, the sleep-wake cycle, learning/memory, anxiety, pain, mood, and brain metabolism (Bear, Connors et al. 2001). However, as suggested earlier, some broad groupings can be determined. The amygdala, hippocampus, and frontal cortex are involved in the cognitive aspects of drug addiction. For example, knowing where to obtain the drug, knowing how to take it, or where the drug paraphernalia is hidden (Da Costa Gomez, Chandler et al. 1996; Bear, Connors et al. 2001; Manning, Merin et al. 2001). The hypothalamus, LC, amygdala, N. raphe magnus, PAG, SC, substantia nigra, and thalamus are all involved in autonomic, physiological, and behavioral effects of opiate intake (Barr and Rossi 1992c; Maldonado, Stinus et al. 1992c; Stornetta, Norton et al. 1993; Tortorici, Robbins et al. 1999; Bear, Connors et al. 2001; Rodriguez Parkitna, Bilecki et al. 2004; Bie and Pan 2005). For instance, changes in respiration, physical withdrawal behaviors, or behaviors that aid in obtaining the drug. And finally there is the NAcc, substantia nigra, and VTA, which each play a role in the rewarding aspects of the drug (encouraging continued intake) and are all part of the

mesolimbic DA system (Koob, Maldonado et al. 1992; Barr and Rossi 1992c; Badiani, Leone et al. 1995; Leshner 1996; Bear, Connors et al. 2001; Gracy, Dankiewicz et al. 2001; Walters and Blendy 2001; Chartoff, Papadopoulou et al. 2003).

For the purposes of this dissertation, four major CNS sites (LC, NAcc, PAG, and SC) have been singled out to focus on because this laboratory has experience working with them and knows they are functional by 7 days postnatally. The LC, the NAcc, the PAG, and the SC are all prominent areas that show changes in cellular activity when directly (eg. microinfusions) or indirectly (eg. systemic or intracerebroventricular injections) exposed to opiates in both adult and infant animals (Maldonado, Fournie-Zaluski et al. 1992a; Maldonado, Stinus et al. 1992c; Guitart and Nestler 1993; Granados-Soto, Kalcheva et al. 2000; Jones and Barr 2001; Maeda, Kishioka et al. 2002).

The Locus Coeruleus. The LC is almost exclusively composed of NEergic neurons (Leshner 1996; McClung, Nestler et al. 2005) and the role of cAMP and its interaction with opiate tolerance and dependence has been extensively studied in this region. Acute administration of opiates inhibits the firing rate of LC neurons and reduces levels of cAMP and PKA (Nestler 2001). Chronic administration of opiates causes the firing rates to return to baseline as the neurons develop tolerance to the opiate (Guitart and Nestler 1993; Nestler 2001; Dang and Williams 2004). During opiate withdrawal firing rates rise above the baseline level (Hayward, Duman et al. 1990; Guitart and Nestler 1993) and when an opiate antagonist is injected into the LC physical withdrawal signs are increased in both adults and infants (Maldonado, Stinus et al. 1992c; Jones and Barr 2001). In mice and rats, both acute and chronic morphine treatment regulate the expression of multiple

genes within the LC, many of which are regulated by cAMP or CREB or contain CRE sites within in their promoter regions (McClung, Nestler et al. 2005).

In addition, protein kinase antagonists microinjected into the LC greatly attenuate the expression of opiate withdrawal (Maldonado, Valverde et al. 1995; Punch, Self et al. 1997). It has been established that the LC is functional by PD7 (Wilson and Leon 1988) and the microinjection of an opiate antagonist into the LC elicits withdrawal in morphine dependent 7-day old rats (Jones and Barr 2001).

The Nucleus Accumbens. As previously mentioned, as one of the major sites that comprise the mesolimbic dopamine system, the NAcc receives DAergic projections from the VTA (Diana, Pistis et al. 1995; Margolis, Hjelmstad et al. 2003) and contains opioid receptors (Koob and Bloom 1988; Chieng and Williams 1998; Shippenberg and Elmer 1998). When injected systemically with acute morphine levels of prodynorphin mRNA increase in the NAcc, when injected chronically levels decrease, and acute withdrawal causes levels to increase beyond control (Przewlocka, Turchan et al. 1996). The findings are similar when looking at local cerebral glucose utilization (LCGU), such that levels of LCGU decrease during chronic treatment (Kimes and London 1989) and increase during acute withdrawal (Wooten, DiStefano et al. 1982). The fact that these tolerance findings are in opposition to that found in the LC is further evidence that the heterogeneity of the CNS makes global assumptions from just one study inappropriate. Although, as evidenced by a study that found an up-regulation of $G_{q/11\alpha}$ and phosphorylated PKC in the NAcc after chronic intermittent morphine treatment (Narita, Mizuo et al. 2002), it may also be the result of different experimental designs. Finally, during precipitated opiate withdrawal, an increase in Fos-LIR and mRNA is found in the NAcc (Hayward, Duman

et al. 1990), which is associated with an increase in conditioned place aversion (Gracy, Dankiewicz et al. 2001).

The Periaqueductal Gray. The PAG is commonly associated with defensive and stereotypic behavior, analgesia, and autonomic regulation (Bandler and Shipley 1994; Tortorici, Robbins et al. 1999; Sim-Selley, Selley et al. 2000; Bagley, Gerke et al. 2005). This group of neurons is in close proximity to the LC and therefore it has been suggested that some of the effects believed to be associated with the LC are in fact the result of neurons in the PAG becoming active (Christie, Williams et al. 1997). Physical dependence can be induced by the chronic administration of analogues for the opioid peptide enkephalin into the PAG (Maldonado, Fournie-Zaluski et al. 1992a), and a pronounced withdrawal syndrome is seen following the direct and systemic administration of various opiate antagonists (Laschka, Teschemacher et al. 1976b; Laschka and Herz 1977; Aghajanian 1978; Hayward, Duman et al. 1990; Maldonado, Fournie-Zaluski et al. 1992a; Chieng, Keay et al. 1995b; Jones and Barr 2001). In addition physical withdrawal can be attenuated by the direct administration of enkephalin catabolism inhibitors into the PAG (Maldonado, Fournie-Zaluski et al. 1992a). At the cellular level, increases in metabolic activity are seen during withdrawal (Kimes and London 1989) as well as increases in Fos-LIR (Chieng, Keay et al. 1995b) and increases in action potential rates (Bagley, Gerke et al. 2005) in the adult rodent. The direct administration of protein kinase antagonists into this area also attenuates opiate withdrawal behaviors (Maldonado, Valverde et al. 1995; Punch, Self et al. 1997) and the increased action potential rate seen during withdrawal (Bagley, Gerke et al. 2005).

The Spinal Cord. The SC is one of the major sites of action of opiates and opioid peptides, with the greatest concentration of opioid receptors located within the dorsal horn (Bear, Connors et al. 2001). For these reasons, the SC is an important anatomical region when studying analgesic tolerance and withdrawal in rodents. During withdrawal, expression of the Fos protein is increased in animals of all ages (Beckmann, Matsumoto et al. 1995; Rohde, Detweiler et al. 1996; McPhie and Barr 2003) in the SC. In addition, chronic intrathecal (i.t.) injections of morphine increase levels of PKC-dependent phosphorylation, as well as PKC-α and PKC-γ immunoreactivity in the dorsal horn of the SC (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000). Acute exposure to morphine blocks calcium entry into the SC (Bernstein and Welch 1995), resulting in a decreased production of cAMP. Finally, pretreatment with an i.t. injection of an opiate antagonist blocks the development of dependence (Delander and Takemori 1983), and the precipitation of withdrawal increases levels of Fos-LIR within the dorsal horn of the SC (Rohde, Detweiler et al. 1996).

In an attempt to determine comparable neuroanatomical sites that become active during opiate withdrawal in the young rodent, chapter 4 explores the localization of Fos expression in the 7-day old rat and chapter 6 explores localized changes in levels of PKA and PKC during precipitated morphine withdrawal.

Development of the Mechanisms Underlying Opiate Tolerance and Dependence

At this time the picture is far from complete, but there is published data on the developmental time courses of some of the major components of the cAMP/AC and IP_3-DAG signal transduction pathways. A vast amount of data has been published on the early appearance of the three opioid receptors (μ, κ, δ) and their roles in pain,

reinforcement, reward, neurotransmitter release, and neuroendocrine modulation (Georges, Normand et al. 1998). The precise appearance of each receptor changes from study to study because an array of techniques have been used, which include autoradiography, membrane binding studies, and in situ hybridization (Kornblum, Hurlbut et al. 1987; Rius, Barg et al. 1991a; Georges, Normand et al. 1998). However, the overall finding is that in the CNS μ- and κ-ORs appear and are functional early during prenatal development, while the δ-OR does not appear and is not functional until later during development (just before birth) (Kornblum, Hurlbut et al. 1987; De Vries, Hogenboom et al. 1990; Rius, Barg et al. 1991a; Georges, Normand et al. 1998; Zhu, Hsu et al. 1998a). In addition, each receptor displays changes in levels and /or in anatomical distribution throughout the CNS from prenatal development to adulthood (Kornblum, Hurlbut et al. 1987; Szücs and Coscia 1990; Zhu, Hsu et al. 1998a).

Opioid receptors are coupled to inhibitory, stimulatory, or neutral intracellular G-proteins. These G-protein families are comprised of multiple members, each identified by the α-subunit that comprises 1/3 of the G-protein heterotrimer. The G_{i1} and G_{i2} are both detected as early as embryonic day (ED) 10 and 14.5, respectively (Rius, Streaty et al. 1991b; Ihnatovych, Novotny et al. 2002a); and $G_{s\text{-}l}$ and $G_{s\text{-}s}$ are detected as early as ED15.5 (Rius, Streaty et al. 1991b; Rius, Mollner et al. 1994; Ihnatovych, Novotny et al. 2002a). To date the functional activity of G-proteins has not been studied earlier than PD1, at which time they have been shown to be active and functional (as evidenced by ADP-ribosylation) (Szücs and Coscia 1990). Although it is worth noting that even by PD7 there is evidence that G-protein coupling to μ-ORs is weaker than that found in adult rodents (Windh and Kuhn 1995a).

The primary effector adenylyl cyclase is detectable and shows enzymatic activity by PD1 (Ihnatovych, Novotny et al. 2002b). In addition, the second messenger cAMP is detectable by ED14.5 (Rius, Mollner et al. 1994). As with many of the other components of the signal transduction pathway, different types and isozymes have been identified for many of the protein kinases. For instance, PKC can be further divided into type I, II, and III (Hashimoto, Ase et al. 1988), as well as three isozyme groups Ca^{2+}-dependent (α, βI, βII, γ), Ca^{2+}-independent (δ, ε, η, θ), and atypical (ζ, λ) (Jiang, Naik et al. 1994; Narita, Mizuo et al. 2002). Each one of the PKCs has a slightly different developmental profile and CNS distribution, but they are all present by birth (PKCζ is detectable as early as ED18 (Jiang, Naik et al. 1994)) (Hashimoto, Ase et al. 1988; Herms, Zurmohle et al. 1993; Jiang, Naik et al. 1994; Lee, Kato et al. 1994), with the exception of type I which is not detectable until after birth (Hashimoto, Ase et al. 1988; Herms, Zurmohle et al. 1993; Jiang, Naik et al. 1994; Lee, Kato et al. 1994). Other PKs that are present by birth include Ca^{2+}/calmodulin-dependent PK (CaMKII) (Herms, Zurmohle et al. 1993) and phospholipid-sensitive Ca^{2}-dependent PK (PL-Ca-PK) (Turner, Raynor et al. 1984). Finally, there is no second messenger pathway that would be complete without mention of the development of transcription factors that play a critical role in the modulation of gene transcription and protein synthesis. High levels of CREB immunoreactivity are detectable as early as PD7, returning to the low basal levels commonly seen in adult brains by PD21 (Pennypacker, Hudson et al. 1995). (See Table 2-2).

Despite the different techniques and different prenatal and postnatal ages that each researcher has investigated the overall picture is that, with the exception of CREB, all components of the various second messenger pathways, that have been studied to date,

are present and functional by birth. Overall, their pattern is to emerge in high levels during early development and gradually decrease to the low levels typically found during adulthood. It is also important to note that there is evidence that even though some of the components of the second messenger pathways are present and functional they are still not fully mature and do not function in the same manner in which they function in the adult. As a result, it has been theorized that in the neonatal rat the δ-OR contains structural features that are unique to that age, or that the neonatal opioid receptor sites have not been completely gylcolysized, all of which result in functioning that is different from the mature δ-ORs found in the adult rodent (Szücs and Coscia 1990). Therefore it is possible that a drug treatment meant to alleviate withdrawal behaviors in an infant will not have the same effect as it does in an adult because the underlying mechanisms that the drug is meant to target are not fully developed and/or functional.

Mechanisms Underlying Opiate Tolerance and Dependence in the Young Rodent

The first five sections of this chapter were intended as a brief introduction to the literature exploring the signal transduction systems that underlie opiate tolerance and dependence in the adult. In addition, at the end of certain sections, the chapter of this dissertation that describes an experiment meant to explore the related area in the infant rat is referenced. There still remains, however, the need for a brief introduction into the current literature on opiate tolerance and dependence and signal transduction pathways during early ontogeny.

There are a handful of studies that explored at the immediate effects of opiate exposure on cellular activity and, as with the adult literature, the findings were contradictory. Both pre- and postnatal opiate treatment reduce μ-OR binding density (Tempel, Habas et

al. 1988; Belcheva, Bohn et al. 1998) and up-regulate κ-OR binding density (Belcheva, Bohn et al. 1998), effects that are gone by PD14, without changing mRNA levels of the μ- and κ-ORs (Belcheva, Bohn et al. 1998). In addition, at PD14 the chronic gestational treatment of the opiate peptide β-endorphin up-regulates μ-ORs, while chronic postnatal treatment down-regulates μ- and δ-OR levels (Zadina, Kastin et al. 1985).

Modeled from adult experiments, there are a few studies that focus on the signal transduction systems and opiate treatment. Chronic prenatal treatment with morphine causes a significant increase in AC activity at gestational day 21 without effecting levels of cAMP (De Vries, Van Vliet et al. 1991), an unexpected finding since cAMP is detectable by this age and directly affected by AC activity. The suppression of slow ventral root potentials (sVRP) by acute morphine at PD7 is blocked by the co-administration of an antagonist for PKCε. Finally, at postnatal day 7 there is a significant increase in *c-fos* mRNA in morphine dependent rats experiencing precipitated withdrawal (Maeda, Kishioka et al. 2002).

Other studies treat the dams during late gestation but do not look at the offspring until early adulthood, where the long-term effects of early opiate exposure can be assessed. Prenatal morphine exposure increases κ-OR density in the FC and POA (Rimanoczy, Slamberova et al. 2001) and δ-OR density in the FC (Vathy, Rimanoczy et al. 2000), while decreasing μ-OR density in the POA of rats during early adulthood (Rimanoczy and Vathy 1995). Prenatal heroin exposure does not alter the activity level of the α-subunit of the G-protein in the hippocampus (Zamir and Yanai 1995), but it does increase the activity level of the whole G_i- and G_q-proteins in the hippocampus of adult mice (Steingart, Barg et al. 1998; Yanai, Steingart et al. 2000; Yanai, Huleihel et al. 2003). In

addition, at postnatal day 50 and 102 prenatal heroin exposure increases K^+-induced IP formation in the hippocampus, an effect that can be reversed by the grafting of new neuronal tissue into that area (Steingart, Barg et al. 1998; Yanai, Steingart et al. 2000). Finally, prenatal heroin exposure in mice causes enhanced activity of stimulated AC in multiple brain regions from postnatal day 4 into early adulthood (Slotkin, Seidler et al. 2001).

Interestingly enough, the findings for PKC have been rather contradictory. The effects on PKC appear to be dependent on the isoforms, as is the case in adults who are exposed during adulthood (Busquets, Escriba et al. 1995; Mao, Price et al. 1995; Garcia-Sevilla, Ventayol et al. 1997; Granados-Soto, Kalcheva et al. 2000). Mice at PD50 who are exposed to heroin from GD9-18 show up-regulation of basal levels for whole PKC (Steingart, Barg et al. 1998; Yanai, Steingart et al. 2000), without effecting activity in either the membrane or cytosol (Steingart, Barg et al. 1998) of the hippocampus. However with the same treatment, basal levels of the PKC isoforms PKCγ and PKCα remain unaffected (Shahak, Slotkin et al. 2003) while the translocation of PKCβII and PKCγ from the cytosol to the membrane is completely prevented (Yaniv, Naor et al. 2004).

Acute Opiate Effects on the cAMP/AC Pathway

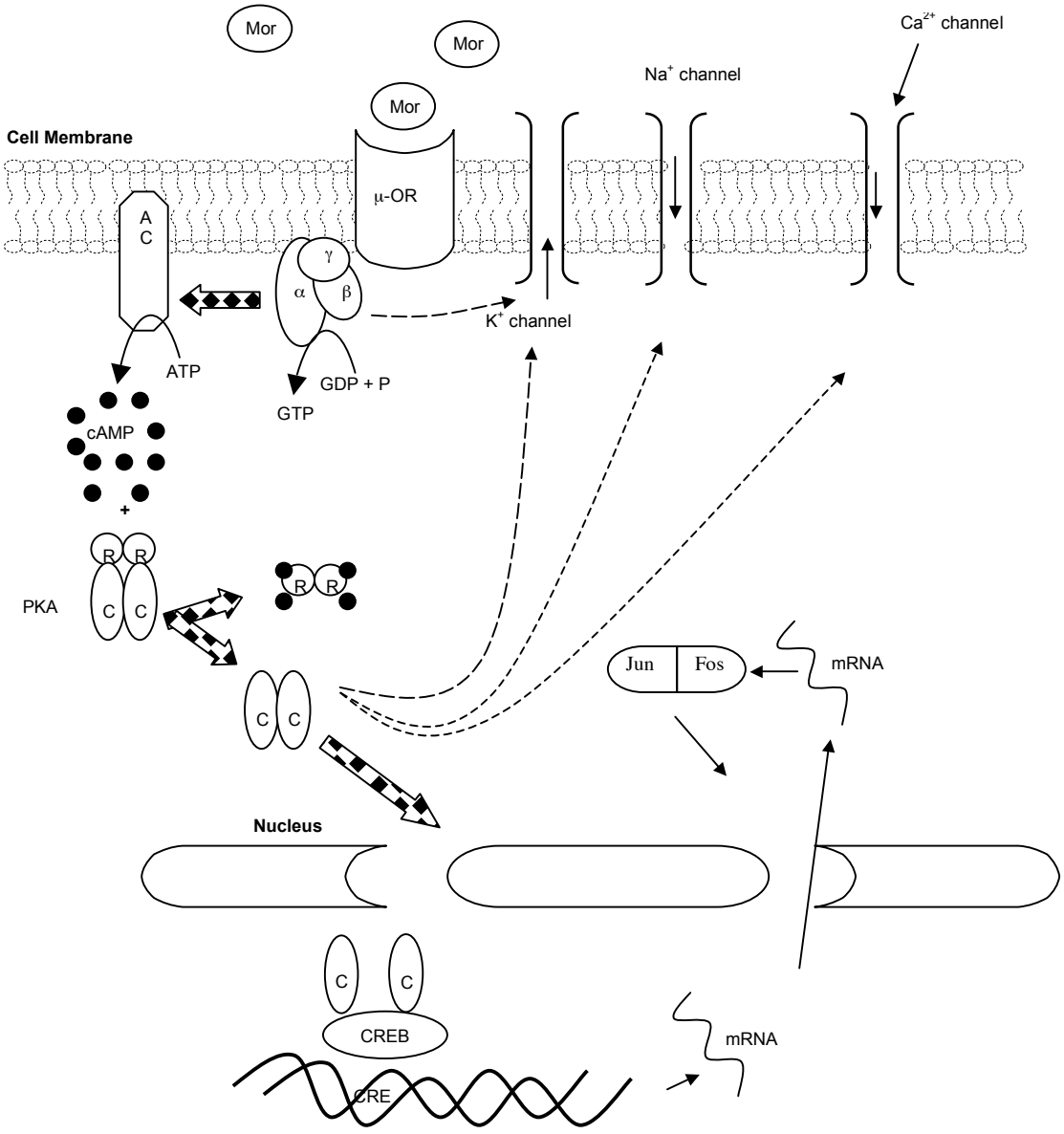


Fig. 2-1. Simplified diagram of the intracellular events that occur in the cAMP/adenylyl cyclase signal transduction pathwa, after an opiate has bound to the receptor. The large patterned arrows show the direct cascades. The dashed arrows show other effects of activation of this second messenger system. **Abbreviations: AC**, adenylyl cyclase; **Ca^{2+}**, calcium; **cAMP**, cyclic adenosine 3',5'-monophosphate; **CRE**, cAMP responsive element; **CREB**, cAMP responsive element binding protein; **K^+**, potassium; **Na^+**, sodium; **-OR**, opioid receptor; **PK**, protein kinase; **R**, regulatory subunit; **C**, catalytic subunit; **α, γ, and β**, G-protein subunits; **Mor**, morphine.

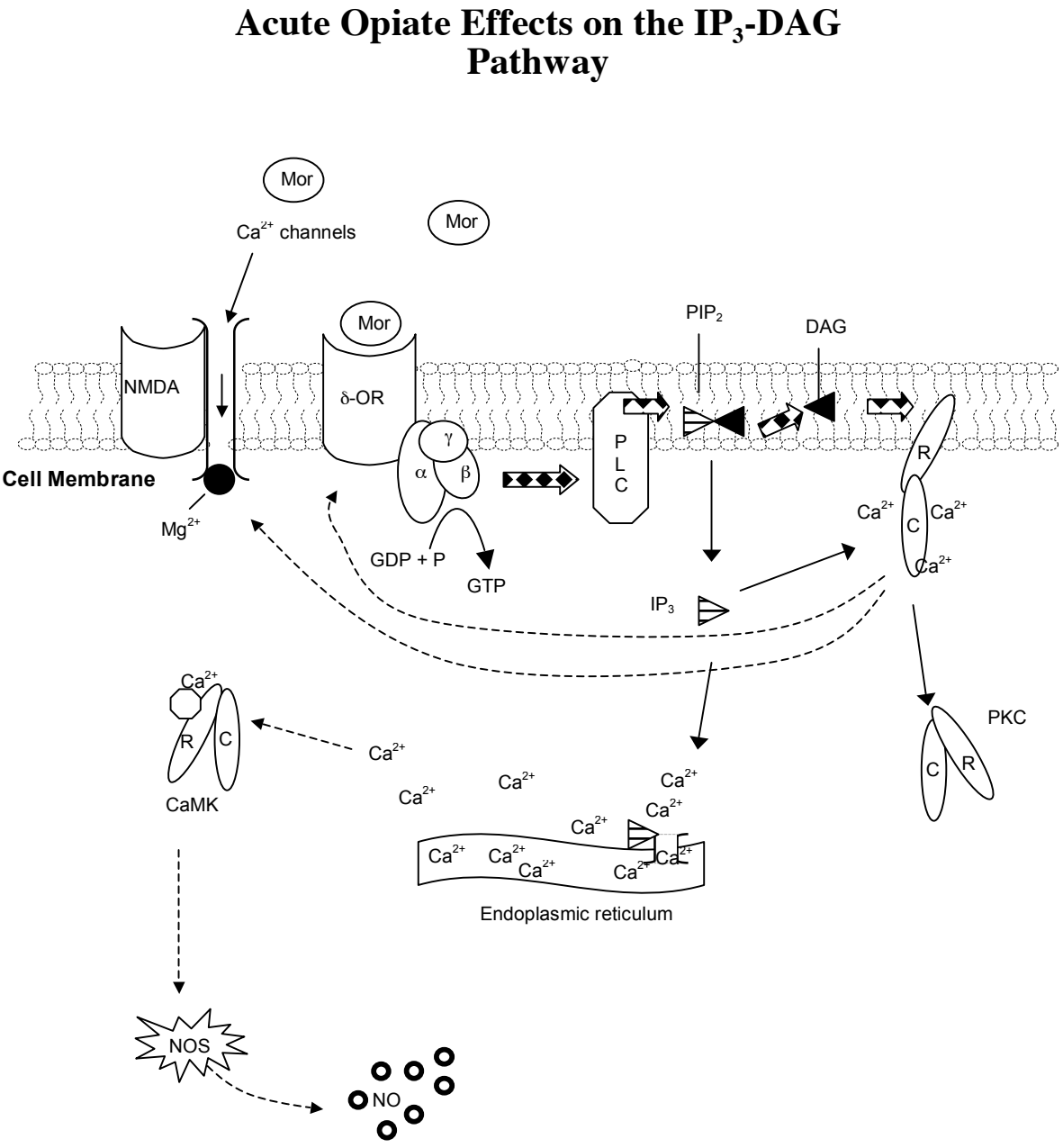


Fig. 2-2. Simplified diagram of the intracellular events that occur in the IP_3-DAG signal transduction pathway, after an opiate has bound to the receptor. The large patterned arrows show the direct cascades of events. The dashed arrows show all other effects of activation of the second messenger system. **Abbreviations**: **Ca^{2+}**, calcium; **CAMKII**, Ca^{2+}/calmodulin protein kinase; **DAG**, diacylglycerol; **IP_3**, inositol 1,4,5-triphosphate; **NMDA**, N-methyl-D-aspartate; **δ-OR**, opioid receptor; **PK**, protein kinase; **R**, regulatory subunit; **C**, catalytic subunit; **NOS**, nitric oxide synthase; **NO**, nitric oxide; **PIP_2**, phosphatidylinositol 4-monophosphate; **PLC**, phospholipase C; **α, γ, and β**, G-protein subunits; **Mor**, morphine.

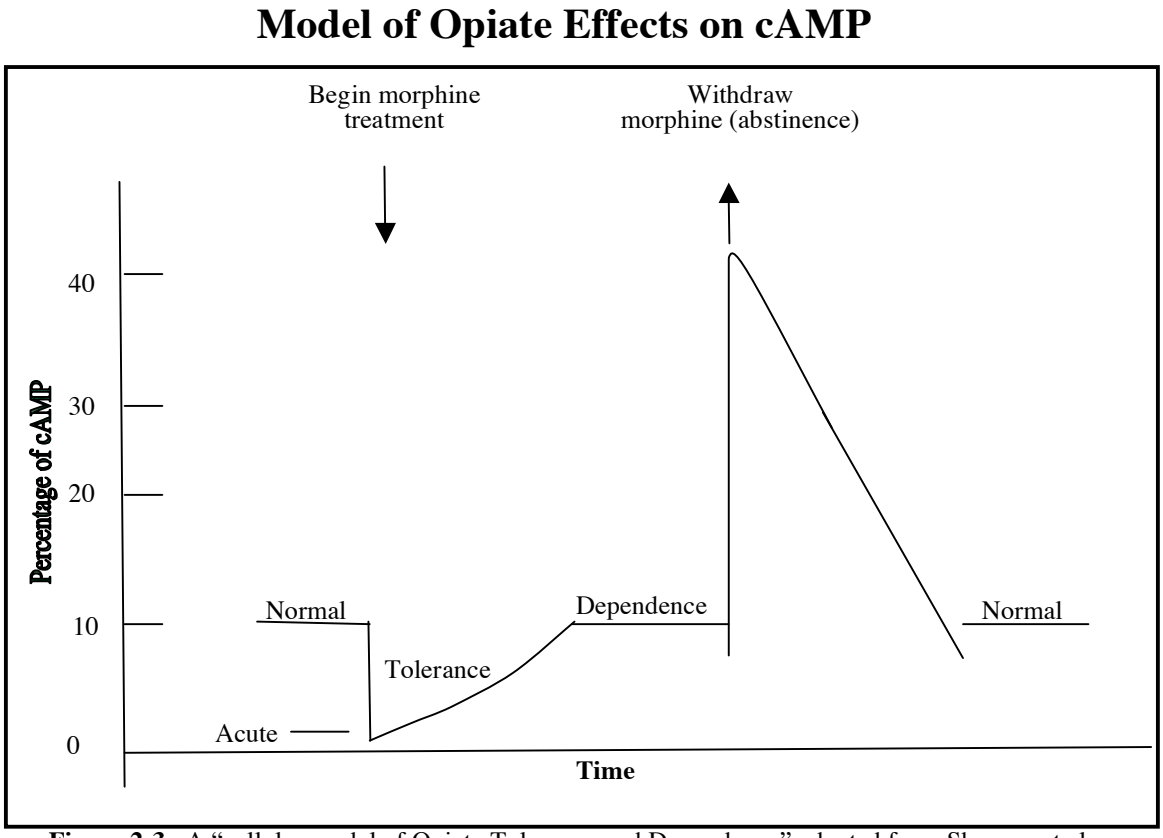


Figure 2-3. A "cellular model of Opiate Tolerance and Dependence" adapted from Sharma, et al (Sharma, Klee et al. 1975) showing the effects of different schedules of morphine treatment on the levels of the second messenger cAMP.

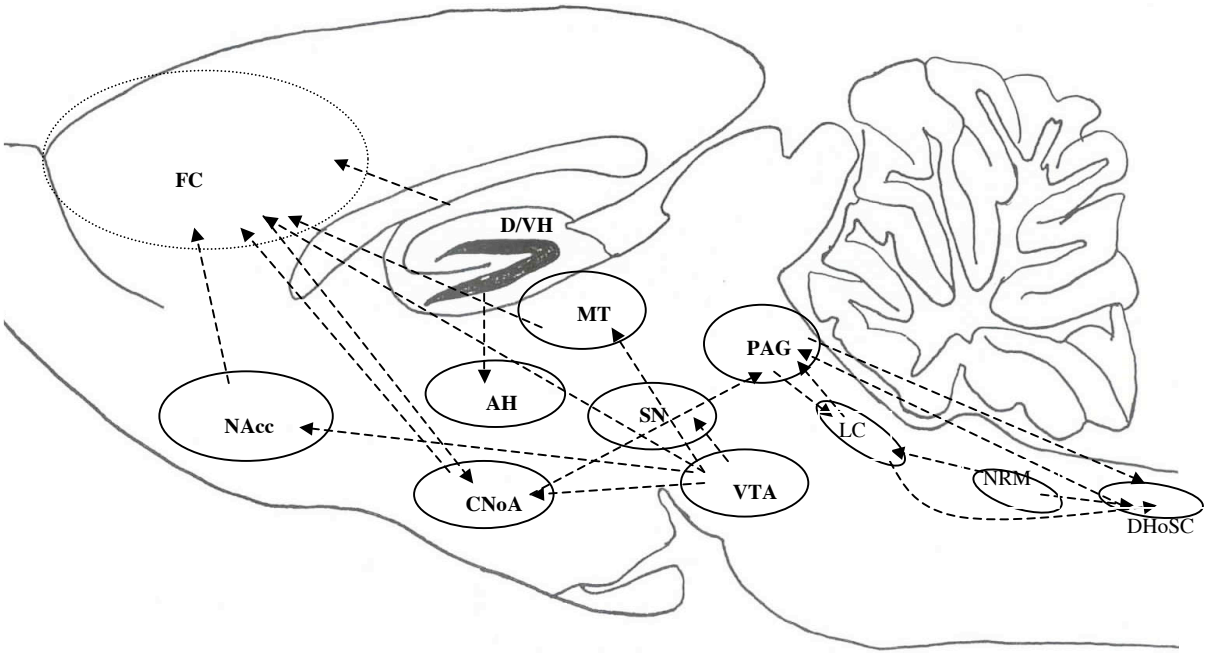


Figure 2-4. Midsagital view of the major brain regions involved in opiate tolerance and dependence. **Abbreviations**: **AH**, anterior hypothalamus; **CNoA**, central nucleus of amygdala; **D/VH**, dorsal and ventral hippocampus; **DHoSC**, dorsal horn of spinal cord; **FC**, frontal cortex; **LC**, locus coeruleus; **MT**, medial thalamus; **NAcc**, nucleus accumbens; **NRM**, nucleus raphe magnus; **PAG**, periaqueductal gray, **SN**, substantia nigra; **VTA**, ventral tegmental area.

Opiate Effects on Different Brain Regions

Site / Effect	Description of effect	References
Amygdala (central nucleus)		
↑,↔	MWS after local injection of opiate antag	(Calvino, Lagowska et al. 1979; Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c; Jones and Barr 2001)
↑	CPA after local injection of opiate antag	(Stinus, Le Moal et al. 1990)
↑	Fos-LIR and mRNA; LCGU and SCFR after W/D	(Wooten, DiStefano et al. 1982; Freedman and Aghajanian 1985; Hayward, Duman et al. 1990; Gracy, Dankiewicz et al. 2001)
↓	LCGU and SCFR after chronic Mor tx	(Freedman and Aghajanian 1985; Kimes and London 1989)
Frontal cortex		
↑	Fos-LIR and mRNA; PKCαβ-LIR after W/D	(Hayward, Duman et al. 1990; Busquets, Escriba et al. 1995)
↓	PKCαβ-LIR after chronic Mor	(Busquets, Escriba et al. 1995)
↑	*c-fos* mRNA after chronic Mor trx	(Erdtmann-Vourliotis, Mayer et al. 1998)
↑/↓	Gene expression during chronic Mor and W/D	(Ammon, Mayer et al. 2003)
Hippocampus (ventral and dorsal)		
↓, ↑	LCGU after chronic Mor tx	(Kimes and London 1989)
↓	LCGU after nalox W/D	(Wooten, DiStefano et al. 1982)
↔	Fos-LIR and mRNA after naltrex W/D	(Hayward, Duman et al. 1990)
Hypothalamus (anterior)		
↑	MWS elicited by microinjection of Mn	(Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c)
↑	Fos-LIR after naltrex W/D	(Stornetta, Norton et al. 1993)
↓	LCGU after chronic Mor tx	(Kimes and London 1989)
Locus coeruleus		
↑	MWS and SCFR after admin of opiate antag	(Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c; Aston-Jones, Hirata et al. 1997; Jones and Barr 2001)
↔,↑	Fos-LIR and mRNA after nalox W/D	(Hayward, Duman et al. 1990; Stornetta, Norton et al. 1993; Chieng, Keay et al. 1995b)
↓	LCGU after chronic Mor tx	(Kimes and London 1989)
↓	Firing rate with acute Mor	(Zhu and Zhou 2001c)
↑/↓	Gene expression during chronic Mor and W/D	(McClung, Nestler et al. 2005)
Nucleus accumbens		
↑	MWS and CPA after local injection of opiate antag	(Stinus, Le Moal et al. 1990; Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c; Gracy, Dankiewicz et al. 2001)
↑	Fos-LIR and mRNA after antag W/D	(Hayward, Duman et al. 1990; Gracy, Dankiewicz et al. 2001)

↓	LCGU after chronic Mor tx	(Kimes and London 1989)
↑	LCGU after nalox W/D	(Wooten, DiStefano et al. 1982)
↑	Phosphorylated CREB levels during W/D	(Chartoff, Papadopoulou et al. 2003)
N. raphe magnus		
↑	MWS elicited by microinjection of Mn	(Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c)
↑	Fos-LIR after naltrex W/D	(Stornetta, Norton et al. 1993)
Periaqueductal gray		
↑	MWS elicited by microinjection of Mn	(Laschka and Herz 1977; Bozarth and Wise 1984; Stinus, Le Moal et al. 1990; Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c)
↔	Lever pressing for intracranial Mor	(Bozarth and Wise 1984)
↑	CPA after local injection of Mn	(Stinus, Le Moal et al. 1990)
↑,↔	Fos-LIR and mRNA after antagonist W/D	(Hayward, Duman et al. 1990; Chieng, Keay et al. 1995b)
↑	Rate of action potential during W/D	(Bagley, Gerke et al. 2005)
Spinal cord (dorsal horn)		
↑	MWS from IT nalox tx	(Wongchanapai, Tsang et al. 1998)
↑	Fos-LIR of opiate tolerant rats	(Rohde, Detweiler et al. 1996)
↑	PKCαγ- and PKCγ-LIR after chronic Mor	(Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000)
Substantia nigra		
↓	LCGU after chronic Mor tx	(Kimes and London 1989)
Thalamus (medial)		
↑	MWS by microinjection of Mn	(Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c)
↑	CPA after local injection of Mn	(Stinus, Le Moal et al. 1990)
↓	LCGU after chronic Mor tx	(Kimes and London 1989)
↑	LCGU after nalox W/D	(Wooten, DiStefano et al. 1982)
Ventral tegmental area		
↑	Lever pressing for intracranial Mor	(Bozarth and Wise 1984)
↔,↑	MWS after antagonist	(Bozarth and Wise 1984; Stinus, Le Moal et al. 1990)
↑	CPA and CPP from injection of Mor into VTA	(Stinus, Le Moal et al. 1990; Barr and Rossi 1992c)
↑	Fos-LIR and mRNA after naltrex W/D	(Hayward, Duman et al. 1990; Chieng, Keay et al. 1995b)
↓	LCGU after chronic Mor tx	(Kimes and London 1989)
↑/↓	Gene expression during chronic Mor and W/D	(McClung, Nestler et al. 2005)

Table 2-1. The activity seen in some of the major neuroanatomical sites that have been studied as they relate to opiate tolerance and dependence. **Abbreviations**: ↓, decrease; ↔, no change; ↑, increase; **SCFR**, single cell firing rate; **MWS**, morphine withdrawal syndrome; **LCGU**, local cerebral glucose utilization; **Mn**, methylnaloxonium; **CPP**, conditioned place preference; **CPA**, conditioned place aversion; **-LIR**, like immunoreactivity; **Antag**,, antagonist; **W/D**, withdrawal; **Mor**, morphine; **Naltrex**, naltrexone; **IT**, intrathecal.

Development of Different Proteins

Protein/Age	Description of effect	References
Opioid Receptors		
ED11.5	κ- and μ-OR mRNA detected	(Zhu, Hsu et al. 1998a)
ED11.5	Met-enkephalin, dynorphin, β–endorphin immunoreactivity	(Rius, Barg et al. 1991a)
ED12.5	μ-OR detected	(Rius, Barg et al. 1991a)
GD13	mRNA detected	(Georges, Normand et al. 1998)
ED13.5	δ-OR mRNA detected	(Zhu, Hsu et al. 1998a)
ED14.5	κ-OR detected	(Rius, Barg et al. 1991a)
ED17	μ-OR released NA	(De Vries, Hogenboom et al. 1990)
GD21	δ-OR mRNA detected	(Georges, Normand et al. 1998)
PD1	δ-OR detected	(Rius, Barg et al. 1991a)
PD1	κ-OR released DA and NA	(De Vries, Hogenboom et al. 1990)
PD3	κ- and μ-OR detected	(Kornblum, Hurlbut et al. 1987)
PD6	δ-OR detected	(Kornblum, Hurlbut et al. 1987)
PD7	δ-OR released Ach	(De Vries, Hogenboom et al. 1990)
G-proteins		
ED10	G_{i1} and G_o detectable	(Rius, Streaty et al. 1991b)
ED14.5	$G_{s\text{-}l}$ and $G_{s\text{-}s}$ detectable	(Rius, Streaty et al. 1991b)
ED14.5	G_{i2} detectable	(Rius, Streaty et al. 1991b)
PD0	ADP-ribosylation of G_i/G_o α-subunit	(Szücs and Coscia 1990)
PD1	$G_i\alpha$ and $G_s\alpha$ families detectable	(Ihnatovych, Novotny et al. 2002a)
PD1	$G_{s\text{-}l}$ and G_{olf} detectable	(Rius, Mollner et al. 1994)
Primary Effectors		
PD1	ACI, ACII, IV, and VI detectable	(Ihnatovych, Novotny et al. 2002b)
PD1	Low AC enzymatic activity	(Ihnatovych, Novotny et al. 2002b)
Second Messengers		
E14.5	cAMP detectable	(Rius, Mollner et al. 1994)
Protein Kinases		
PD0	PKC: types II and III detectable	(Hashimoto, Ase et al. 1988)
PD0	PKCγ mRNA barely detectable	(Herms, Zurmohle et al. 1993)
PD0	CAM-KIIα mRNA detectable	(Herms, Zurmohle et al. 1993)
PD0	Low detection of PKCα, βI, βIIγ, ε, η	(Jiang, Naik et al. 1994)
PD0	PKCζ, δ, PKMζ are detectable	(Jiang, Naik et al. 1994)
PD0	PL-Ca-PK detectable	(Turner, Raynor et al. 1984)
PD7	PKC: type I detectable	(Hashimoto, Ase et al. 1988)
Transcription Factors		
PD7	CREB factor immunoreactivity in AP-1	(Pennypacker, Hudson et al. 1995)
PD7	CRE DNA binding activity	(Pennypacker, Hudson et al. 1995)

Table 2-2. Development time-line of the components of the cAMP/AC and IP_3-DAG signal transduction pathway. **Abbreviations**: **-OR**, opioid receptor; **GD**, gestational day; **ED**, embryonic day; **PD**, postnatal day; **DA**, dopamine; **NA**; noradrenalin; **Ach**, acetylcholine; **AC**, adenylyl cyclase; **CAM-KII**, Ca^{2+}/calmodulin protein kinase; **PL-Ca-PK**, phospholipid-sensitive Ca^{2+}-dependent protein kinase; **AP-1**, activator protein-1; **CRE**, cAMP responsive element; **CREB**, cAMP responsive element binding protein.

Part Three: Specific Aims

Introduction

Prolonged and frequent exposure to opiates results in the development of tolerance and dependence. Their expression has made the prolonged and extensive use of opiates in clinical pain management problematic. For this reason, the need to create non-addictive drugs, that are able to mimic the desired characteristics of opiates, and/or find drugs that will prevent the expression of opiate tolerance and dependence has spurred an interest in understanding the underlying molecular and cellular mechanisms that are involved in tolerance and dependence.

A current focus has been on the intracellular events that occur as a result of repeated exposure to drugs. These changes are regulated by various signal transduction pathways, that include the IP_3-DAG and cAMP/adenylyl cyclase pathways. Chronic opiate exposure, as well as precipitated withdrawal modulates levels and/or activity of opioid receptors, G-proteins, second messengers, protein kinases, transcription factors, and genes. In addition, pharmacologically or genetically blocking the activity of opioid receptors, protein kinases, and transcription factors alters the expression and development of behavioral tolerance and dependence.

Research on the different signal transduction pathways has focused almost exclusively on the adult rodent and little is known about the infant rodent; despite the fact that over the past several years a number of laboratories has shown that infant rodents are capable of experiencing opiate induced tolerance and dependence (Van Praag and Frenk 1991; Barr and Wang 1992d; Jones and Barr 1995; Windh, Little et al. 1995b; Thornton, Wang et al. 1997). Studying the infant system is especially important because there is evidence

that the fundamental biochemical mechanisms that underlie opiate tolerance and dependence are both different and similar when comparing the adult rodent with the infant rodent. For instance, throughout ontogeny *cfos* mRNA expression is increased during precipitated withdrawal in similar CNS sites (Maeda, Kishioka et al. 2002) and nitric oxide synthase inhibitors decrease the expression of morphine withdrawal (Zhu and Barr 2000). However, the attenuation of morphine withdrawal and tolerance by NMDA receptor antagonists (Zhu and Barr 2001a; Zhu and Barr 2003a) and the precipitation of withdrawal by the intra-amygdala injection of an opiate antagonist in the adult rodent, is not seen in the infant rodent (Jones and Barr 2001).

This lack of data cannot be ignored because prenatal and early postnatal rodent models are indispensable to our understanding of the effects of long-term opiate exposure on human infants and toddlers. Therefore the purpose of the experiments described in this dissertation was to understand the relationship between the IP_3-DAG and cAMP/adenylyl cyclase signal transduction pathways and opiate induced tolerance and dependence during the early postnatal development (7 and 21 days after birth) of the rat.

The overriding hypothesis was that as the infant rat matures, the various components of the signal transduction pathways (eg., receptors, protein kinases, and genes) will play a greater role in regulating opiate tolerance and dependence. To test this hypothesis, experiments were designed to focus on:

1. Opioid receptors (μ-, κ-, and δ-OR)
2. Gene expression (*c-fos* gene)
3. Protein kinases (PKA and PKC)

Specific Aims

Specific Aim 1. To determine which receptor class(es) modulates the distinct withdrawal behaviors seen at PD7. This was determined by the acute pharmacological blockade of the individual receptor types and the subsequent assessment of the expressed withdrawal behaviors.

> *Hypothesis 1.* As is the case in the adult, the acute administration of a μ-OR antagonist will play the greatest role in the expression of morphine withdrawal behaviors.

Specific Aim 2. To determine if distinct neuroanatomical regions show *cfos* (Fos-LIR) activity during morphine withdrawal at PD7. This was determined by the pharmacological precipitation of morphine withdrawal followed by the quantification of Fos-like immunoreactivity (the protein product of *cfos*) using immunohistochemistry.

> *Hypothesis 2.* Despite the relative immaturity of the CNS at PD7, *some* level of activity will be seen in the targeted brain loci (the periaqueductal gray area, nucleus accumbens, spinal cord, and locus coeruleus) during morphine withdrawal.

Specific Aim 3a. To determine how behavioral tolerance and dependence are affected by protein kinase activity during early postnatal ontogeny. This was determined by the acute pharmacological blockade of protein kinase A and protein kinase C and the subsequent assessment of the degree to which withdrawal behaviors and analgesic tolerance were expressed.

Hypothesis 3a. The pharmacological blockade of PKA and PKC activity will show a modulatory effect on the expression of morphine tolerance and dependence at PD21, but not at PD7.

Specific Aim 3b. To determine whether protein levels of PKA and PKC are affected by acute and chronic morphine treatment, as well as precipitated withdrawal during early ontogeny. This was determined by different treatment intervals of morphine and the subsequent quantification of protein levels of PKA and PKC using quantitative western analysis.

Hypothesis 3b. The different treatment intervals of morphine exposure will modulate protein levels of PKA and PKC at PD21, but not at PD7.

Part Four: General Methods

Materials/Methods

All procedures for this study included adequate measures to minimize pain or discomfort and were conducted in accordance with the guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the internal IACUC of Hunter College, CUNY.

Subject*s*

The subjects were the 7- and 21-day old offspring of Long-Evans Hooded rats (Harlan Sprague Dawley) bred and reared in the Hunter College colony room. The colony room was maintained at a constant temperature and humidity level under a light cycle of 12 h, with lights turned on at 08:00h. The dams and their litters were housed in plastic tubs with beta chips used as bedding, and food (Lab Diet) and water available ad libitum. Tubs were checked twice daily (≈ 09:00 and 17:00h) and any new pups found at either time were recorded as 0 days old. The experiment began on PD1 or PD15, and at that time the litter was culled to an appropriate size as determined by the specific experiment.

Tattooing

All pups were permanently tattooed for long-term identification purposes. Based on the Geller and Geller (Geller and Geller 1966) technique, at PD1 pups were removed from the dam and transferred to a smaller plastic container, containing beta chips from the tub of the dam. Using India ink, each pup was permanently tattooed on the ventral surface of between one and four of its paws, depending on the code number.

Drug Preparation

With the exception of chronic morphine, all drugs were assigned randomly and the experimenter was blind to the drug with which each pup was treated. All drugs were reconstituted and/or diluted using dH_2O, with the exception of KT5720, which was reconstituted and diluted with DMSO. All drugs were mixed at a volume of 0.1ml/kg.

Treatment Schedules

Acute treatment. At PD7 or 21 two pups with, no previous exposure to any drug, were removed from the litter and placed in a plastic tub. One pup was weighed and injected with saline (i.p.) while the other pup was weighed and injected with the opiate agonist morphine sulfate (i.p., 10mg/kg). Pups were returned to the huddle until testing.

Chronic Treatment. For pups tested at 7-days of age the treatment duration was from PD1 to PD7 and for pups tested at 21-days of age the treatment duration was from PD15 to PD21. All treatment was for 6.5 days and occurred twice a day. During the morning treatment, each pup from a litter was weighed and given an injection of either saline (i.p.) or morphine (i.p., 10mg/kg). During the evening treatment, pups were injected only. All chronic treatments were within the same litter, such that all pups within a litter received either only saline or only morphine. Pups were returned to the huddle until each one was injected, at which time they were all returned to the dam.

Withdrawal Precipitation. Four hours after the final morning injection on the PD7 or PD21, all pups were removed from the dam, for the last time, and placed in a plastic container in the testing room. One pup was randomly chosen and removed from its littermates; it was weighed and sexed. The target pup was then injected with either saline

or the antagonist of interest, and returned to the huddle (PD7) or placed in a separate tub with one sedated littermate (PD21).

Behavioral Assessment

Withdrawal. Ten minutes after being returned to their tubs, the withdrawal behavior of the target pup was observed every 15 seconds, for a total of fifteen minutes, using scan sampling methods. For this method the observer had a preprinted sheet of paper with a list of all possible withdrawal behaviors described in rats from PD7 to PD21. When the observer scanned the behavior of the target pup on each 15-second interval, a check was placed next to the behavior(s) that was observed at that particular moment. At PD7, once a pup was observed it was sedated and returned to the huddle in order to maintain a constant litter size. The withdrawal behaviors were based on published data describing the distinct withdrawal signs seen throughout the ontogeny of the rat (Jones and Barr 1995). (Table GM -1).

Tolerance. The warm-water tail flick test was used to assess tolerance in each pup, and it was begun one hour or ten minutes after the H-7 or Chelerythrine and KT5720 injections, respectively. The test consisted of submerging approximately 3/4 of the distal portion of the tail of the target pup in a heated water bath (45°C for PD7 and 49°C for PD21). The latency for the pup to withdraw its tail from the water was measured using a mechanical timer connected to a foot pedal. The test began with a baseline measure (before any administration of morphine) of the latency to remove the tail from the water. Next, each pup was injected with saline (i.p.) and its tail withdrawal latency was immediately recorded. This procedure was repeated four more times with each pup receiving cumulatively higher doses of morphine and the addition of a twenty minute time delay

between morphine injection and testing. For pups tested at PD7 the doses were 0.0, 0.3, 1.0, 3.0, and 10.0mg/kg and at PD21 the doses were 0.0, 1.0, 3.0, 10.0, and 30.0 mg/kg of morphine. To ensure that tissue damage did not occur, there was a 20 second cutoff for removal of the tail from the water bath during each testing session.

Withdrawal Behaviors During Ontogeny

Behavior	Definition
Burrow[21]	Sliding the body under the shavings of the observation chamber.
Grooming[21]	Licking/rotary movement of body, including face washing.
Head Moves[B]	Lateral and rotary motions of the head.
Jumping[21]	Sudden leaping such that all four paws are off the bottom of the chamber.
Moving paws[7]	Continuous movement of the hindpaws without walking.
Quiet[B]	Sedated appearance with no movement.
Straub[B]	Extension of the tail.
Stretching[B]	Extension of trunk, causing body lengthening.
Together[B]	Bodily contact with one or more littermates.
Twisting[7]	To bend into a spiral shape.
Walking[B]	Taking more than one or more steps forward.
Wall climbing[B]	Placing at least two forepaws on the wall of the observation chamber.

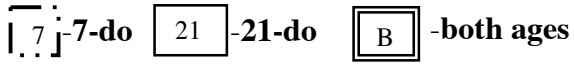


Table GM-1. List and definition of observed physical withdrawal behaviors seen at PD7 and 21. For 15 minutes each animal was observed every 15 seconds and the occurrence of any of the above behaviors was recorded. The information is taken from Jones and Barr, 1995, where it was reported that there are distinct behaviors that occur at PD7 and 21, as well as certain behaviors that appear throughout ontogeny.

Part Five: Where to Start Looking in the Young Rat – Part 1 of Young Rat Experiments

Explanation

Chapter 3

The following chapter sets forth a study that was designed to determine which (if not all) of the major opioid receptor classes (μ-, κ-, and δ-OR) plays a role in opiate withdrawal in the young infant. It was the first attempt to compare the mechanisms that underlie the cellular and molecular changes that occur during chronic opiate exposure in infant rats, to the changes seen in the adult rat. The results of this work were presented at the annual meetings of the Society for Neuroscience (1998) and the International Society for Developmental Psychobiology (1999) and published in 2000 (McPhie and Barr 2000). Minor modifications have been made to it so that it fits in with the broader context of this dissertation. Although the design of the study allowed for only behavioral interpretations it was based on a larger body of work done on the role that the opioid receptors play in the adaptations made after acute and chronic (tolerance and dependence) exposure to opiates. At one time it was theorized that changes in opioid receptor density or function plays a major role in these cellular adaptations to opiates (Trujillo and Akil 1991; Nestler 1992; Sim-Selley, Selley et al. 2000; Liu and Anand 2001; Yoburn, Gomes et al. 2003). However, it has now been well established that while opioid receptors are a crucial step in initiating the cascade of events, other downstream molecules become more important in the long-term maintenance of these alterations in functioning (Trujillo and Akil 1991).

Chapter 4

In an attempt to evaluate another potential area that shows involvement in opiate tolerance and dependence the focus of this chapter was on gene targets of signal

transduction pathways. Immediate early genes (IEGs) are the targets of many cellular cascades of events that occur when a neuron is activated by exposure to opiates. The activation of IEGs as a consequence of certain signal transduction pathways, and their subsequent involvement in protein synthesis, has been theorized to be crucial for the long-term adaptive changes that occur during cellular learning (Kandel, Schwartz et al. 2000). One approach to looking at drug addiction is that it is a form of non-associative learning (Trujillo 1995). That is, at the cellular level, permanent changes occur that cause the neurons to function differently. For instance, after long-term exposure to an opiate, neurons may no longer synthesize proteins with the absence of the opiate. By using Fos, the product of the IEG *c-fos,* as a marker the brain regions that become active during opiate withdrawal were investigated. In addition to being a strong marker for cellular activity, Fos has the ability to regulate the transcription of the three opioid peptides by binding to the AP-1 regulatory elements within their promoter regions (Beckmann, Matsumoto et al. 1995). Therefore this study was expected to help determine two things: 1) Does Fos play a role in opiate withdrawal in the infant, as it does in the adult rat? 2) What are some of the neuroanatomical sites that become active during opiate withdrawal and are they similar to those seen in the adult? It should be noted that a caveat to looking at *c-fos* is that a lack of change in its activity does not necessarily mean that there is no change in overall neuronal activity. There just may be other IEGs involved. The findings of this chapter were presented at the annual meetings of the International Society for Developmental Psychobiology (2000) and the Society for Neuroscience (2001) and submitted to Developmental Brain Research for publication in 2004.

Chapter 3: Receptor Mediation of Tolerance and Dependence

Introduction

Despite the various synthetic and semi-synthetic opiates that have been produced, morphine remains the most commonly used opiate drug in clinical settings for the relief of acute or chronic pain in both adult and infant humans. However, one major disadvantage of morphine is that repeated administration (chronic exposure) often leads to dependence. When dependence occurs, the slow or abrupt (through the administration of an antagonist) cessation of the opiate causes disruption of the basic functioning of the nervous system on different levels (Trujillo and Akil 1991; Nestler 1992; Inturrisi 1999). For instance, at the behavioral level physical withdrawal signs occur; at the physiological level there are changes in body temperature; at the affective level aversive affective states occur; and at the neuronal level there are changes in receptor function.

Human infants, exposed to opiates after in utero or postnatal chronic exposure, experience an array of withdrawal behaviors different from those seen in adults. These behaviors include altered sleep patterns (Dinges, Davis et al. 1980), high-pitched crying, respiratory and gastrointestinal dysfunction, and irritability and tremors (Rajegowda, Glass et al. 1972). In the short-term, dependence and the manifestation of these withdrawal behaviors can result in strained mother-child bonding and increased morbidity or mortality of the infant (Finnegan 1985); the long-term consequences of infant exposure to opiates are still unknown.

In adult rats, opiates exert their pharmacological actions through three opioid receptor classes, μ, κ, and δ, which are phylogenetically conserved. Advances in gene cloning and related techniques have shown that each receptor has its own unique pharmacological profile (with differing degrees of affinity to the endogenous and synthetic opioids), anatomical distribution throughout the central and peripheral nervous system, developmental profile, and function.

In the adult rat, the μ-opioid receptor has been shown to play a vital role in eliciting physical withdrawal. The direct blockade of the μ-opioid receptor, by a specific antagonist or a specific agonist after a non-specific antagonist challenge, results in withdrawal behaviors that are quantitatively greater than those seen from similar experiments with the δ- or κ-opioid receptors (Cowan, Zhu et al. 1988; Stevens and Yaksh 1989; Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b). Withdrawal behaviors associated with the blockade of the δ- and κ-opioid receptors are either less dramatic or are not seen at all (Young and Khazan 1985; Cowan, Zhu et al. 1988; Stevens and Yaksh 1989; Maldonado, Feger et al. 1990; Maldonado, Negus et al. 1992b).

Early experiments focusing on the ability of infant rats to experience withdrawal and dependence to morphine, did not detect any behavioral changes before the age of 30 days (Fanselow and Cramer 1988). However, more recent research has shown that infant rats do in fact experience withdrawal behaviors (Jones and Barr 1995; Windh, Little et al. 1995b). Most of these behaviors change throughout the ontogeny of the rat until the behaviors seen in adulthood are expressed. For instance, behaviors that are commonly seen in adults, like wet dog shakes, grooming, jumping, diarrhea, and ptosis (Jones and Barr 1995; Windh, Little et al. 1995b; Thornton, Wang et al. 1997) do not appear until

21-days of age, whereas at 7-days of age behaviors like paw movements, head movements, and rolling are common (Jones and Barr 1995).

The expression of withdrawal behaviors that are qualitatively different throughout the ontogeny of the rat may be due, in part, to the different development of the CNS μ-, κ-, and δ-opioid receptors. The present study was designed to determine whether the unique withdrawal behaviors seen in the 7-day old infant rat are in fact due to receptor classes distinct from those involved in the display of physical withdrawal behaviors in the adult rat.

In order to determine the specific participation of each opioid receptor type in the expression of morphine abstinence, we quantified the occurrence of a series of different physical withdrawal behaviors precipitated by the administration of the selective μ-opioid receptor antagonist CTOP, the selective κ-opioid receptor antagonist nor-BNI, and the selective δ-opioid receptor antagonist naltrindole. We hypothesized that, as is the case in the adult rat, the μ-opioid receptor plays the greatest role in modulating the withdrawal behaviors seen in the 7-day old rat and is therefore the predominant receptor modulating this behavior throughout the ontogeny of the rat.

Materials and Methods

Subjects/Tattooing

As described in the general methods section.

Establishment of Tolerance

As described in the general methods section.

Withdrawal/Behavioral Observation

As described in the general methods section. Pups were injected with either saline or one of four doses (0.1, 0.3, 1.0, 3.0 μg/4 μl, intracisternal) of D-Phe-Cys-Try-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), naltrindole hydrochloride H_2O (Naltrindole), or nor-binaltorphimine (nor-BNI). The intracisternal (IC) injection procedure was adapted from (Carden, Barr et al. 1991).

Data Analysis

A factorial analysis of variance (ANOVA) was conducted for each behavior. Wall climbing and separated were omitted because they rarely occurred. Each pup was observed for 15 minutes, which was then divided into 3 time periods, consisting of 5 minutes each. Each time period was treated as a within-subjects variable. No significance occurred across the time periods; thus that factor was not included in the analysis. All four doses were injected within a single litter and the drug dose effect was treated as a repeated measures variable.

Results

CTOP

The IC administration of the μ-opioid receptor antagonist CTOP caused withdrawal in the 7-day old rat. Of the observed behaviors, there was a significant increase in the amount of time spent stretching $F(4,28) = 3.2$, $p < .05$; twisting $F(4,28) = 3.7$, $p < .05$; and walking $F(4,28) = 2.9$, $p < .05$; and a decrease in the amount of time spent being quiet $F(4,28) = 4.4$, $p < .05$. The frequency of each of these significant behaviors was dose dependently increased by CTOP (or decreased, in the case of the quiet behavior). The three remaining observed behaviors, head moves, moving paws, and together showed no significant change in the number of occurrences. See Figure 3-1.

Naltrindole and nor-BNI

Neither the IC administration of the specific δ-opioid receptor antagonist naltrindole nor the specific κ-opioid receptor antagonist nor-BNI caused withdrawal behaviors in the 7-day old rat. (See figures 3-2 and 3-3, respectively). There was no significant change in the frequency of occurrences of each of the observed behaviors by either one of the antagonists.

Discussion

The IC administration of the specific μ-opioid receptor antagonist CTOP caused withdrawal in the 7-day old rat, significantly increasing the occurrence of the stretching, twisting, and walking behaviors, while decreasing the occurrence of the quiet behavior. CTOP did not significantly increase the occurrence of the head moves, moving paws, and together behaviors in the infant rats. The IC administration of the specific δ-opioid receptor antagonist naltrindole and the specific κ-opioid receptor antagonist nor-BNI, however, caused no significant changes in the occurrence of any of the observed behaviors in the 7-day old rat.

The μ-opioid receptor: These results are in accordance with published pharmacological findings about the role of the μ-opioid receptor in the withdrawal behaviors displayed by adult rodents. The administration of CTAP (a derivative of the antagonist CTOP), naltrindole, and nor-BNI to morphine dependent adult rats, resulted in the display of withdrawal signs from all three drugs (Maldonado, Negus et al. 1992b). However, the number of signs that were significantly increased by CTAP was far greater than the number of signs increased by either naltrindole or nor-BNI. In addition, when CTAP was combined with either naltrindole or nor-BNI, the degree to which the withdrawal

behaviors significantly increased in appearance was no different from the degree to which the behaviors were elicited by CTAP alone (Maldonado, Negus et al. 1992b). Similar results were found with rats that were chronically administered the agonists DAMGO, DPDPE, and U-50,488H specific to the μ, δ, and κ (respectively) receptor classes. Upon administration of the nonselective opioid antagonist naloxone, the μ–opioid receptor agonist DAMGO yielded the highest abstinence scores (Cowan, Zhu et al. 1988).

MOR (morphine opioid receptor) lacking mice showed none of the usual physical withdrawal signs (jumping, teeth chattering, wet dog shakes, etc.) and no conditioned place aversion, after the administration of naloxone (Matthes, Maldonado et al. 1996). Despite these distinct behavioral differences, the mutant mice showed no difference in the total number and brain distribution of the δ- and κ-opioid receptors, or the expression of mRNA for the endogenous peptide genes, proenkephalin, prodynorphin, and proopiomelenocortin (Matthes, Maldonado et al. 1996). Thus the present data are consistent with the adult rodent data.

The κ-Opioid Receptor: As with the μ-opioid receptor, the κ-opioid receptor is detectable and functional (as measured by its ability to release dopamine) by gestational day 17 (De Vries, Hogenboom et al. 1990) and its mRNA is present by gestational day 13 (Georges, Normand et al. 1998) in the rat. In the mouse, binding (Rius, Barg et al. 1991a) has been found as early as embryonic day 11.5, as well as the presence of mRNA for the κ-opioid receptor (Zhu, Hsu et al. 1998a). In the PD1 (postnatal day 1) rat it is present in high concentrations and increases to reach its adult levels by age 30 (Barr, Paredes et al. 1986; Kornblum, Hurlbut et al. 1987; McDowell and Kitchen 1987). Therefore, the finding that the blockade of the κ-opioid receptor did not result in any

level of increase in the appearance of withdrawal behaviors in the 7-day old rat was unexpected.

The role of the κ-opioid receptor in physical withdrawal behaviors in the adult is less clear. Pharmacological studies have found a more robust physical withdrawal syndrome in morphine dependent adult rats pretreated with a single dose of nor-BNI, as compared to rats pretreated with saline (Suzuki, Narita et al. 1992; Spanagel, Almeida et al. 1994). In addition, the co-administration of U-50, 488H and morphine in adult mice reduced the display of morphine induced locomotor activity and straub tail (Suzuki, Narita et al. 1990). These findings suggest that one of the roles of the κ-opioid receptor in withdrawal may be to antagonize some of the effects of the μ–opioid receptor; although it is not clear if this is also the case in infant rats.

Genetic studies have found the absence of the κ-opioid receptor to play a more direct role in adult withdrawal. In morphine treated KOR (kappa opioid receptor) deficient mice, the occurrence of physical withdrawal signs was significantly lower than that seen in wild-type mice (Simonin, Valverde et al. 1998). As is the case with the MOR deficient mice, no difference was found among KOR deficient, and wild-type mice in terms of their expression of mRNA for the endogenous opioid peptide genes, and the number and distribution of the δ- and μ–opioid receptors. Thus, the role of the κ-opioid receptor, in the morphine abstinence syndrome, is less clearly defined in both the adult and infant rat.

The δ-Opioid Receptor: The δ- opioid receptor protein is not present until at least postnatal days 5-7 (De Vries, Hogenboom et al. 1990; Szücs and Coscia 1990; Oommen and Anand 1995) in the rat, although its mRNA is detectable by gestational day 13.5 in

the mouse (Zhu, Hsu et al. 1998a). Therefore, it was expected to play no role in the abstinence syndrome of the 7-day old rat, as was the case in this study.

Conclusion: As was hypothesized, the μ-opioid receptor plays the greatest role in modulating the withdrawal behaviors seen in the 7-day old rat and is therefore the predominant receptor modulating this behavior throughout the ontogeny of the rat. The appearance of behavioral withdrawal signs like paw movements, head movements, and rolling in the 7-day old rat that are eventually replaced by behaviors like wet dog shakes, grooming, diarrhea, and ptosis, are mediated by the μ-opioid receptor as they are in the adult. However, it is apparent that there are mechanisms secondary to the μ-opioid receptor that play a significant role in the display of the behaviors that are unique to the infant. The nature of these secondary mechanisms is an area that requires exploration. The exact role of the κ- and δ- opioid receptors in dependence and the display of physical withdrawal behaviors is less clear, but the present evidence suggest that the δ- and κ-opioid receptors play no role in the display of the physical opiate withdrawal behaviors in the young rat.

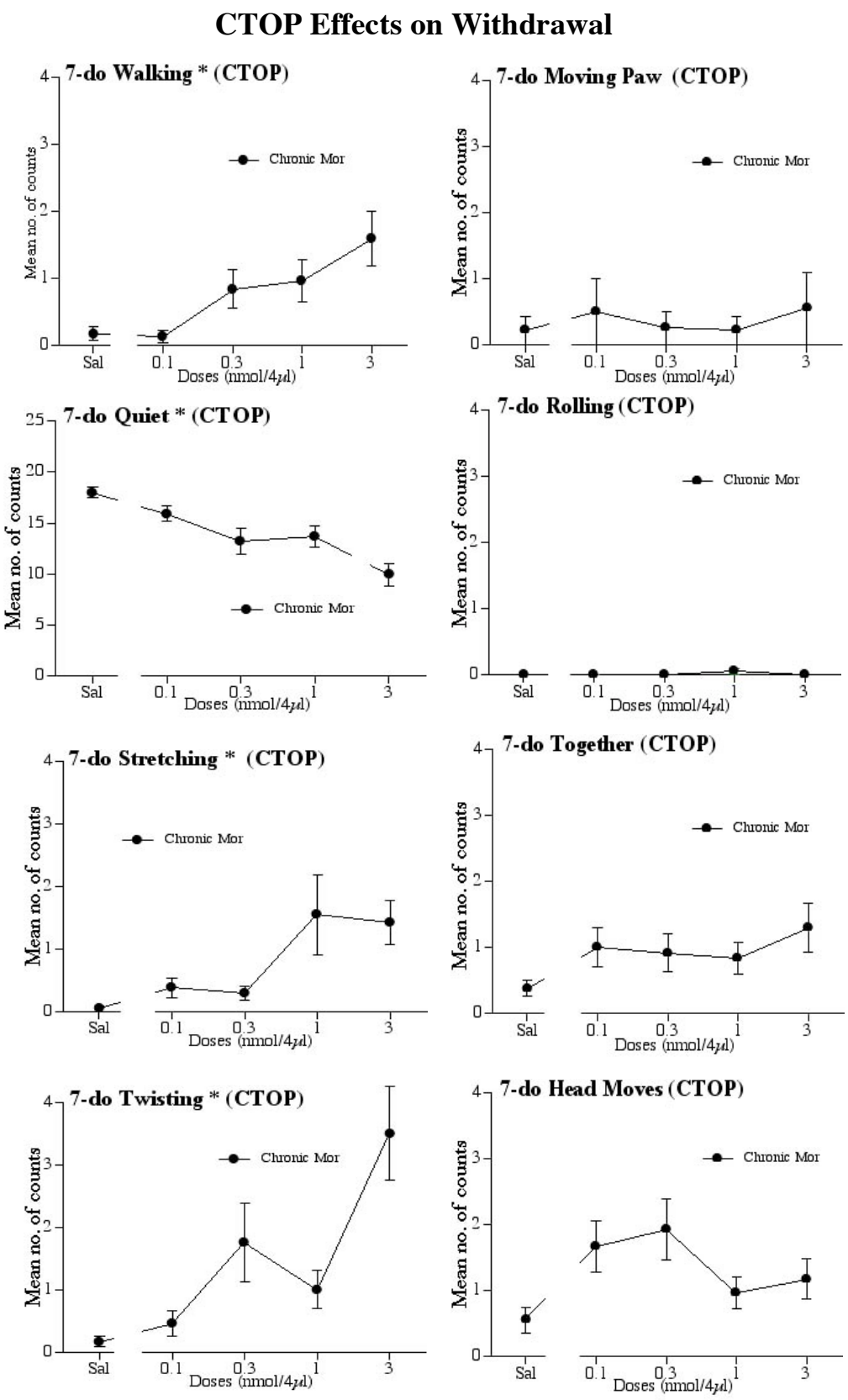


Fig. 3-1. Dose effect of CTOP, the μ -OR antagonist. This figure shows the mean number (± SEM) of occurrences, in 15 minutes, of various morphine withdrawal behaviors in the 7-day old rat. The left column depicts behaviors that showed significant dose effects ($p < 05$). The right column depicts behaviors that showed no significant dose effects. *Quiet is presented on a different scale.

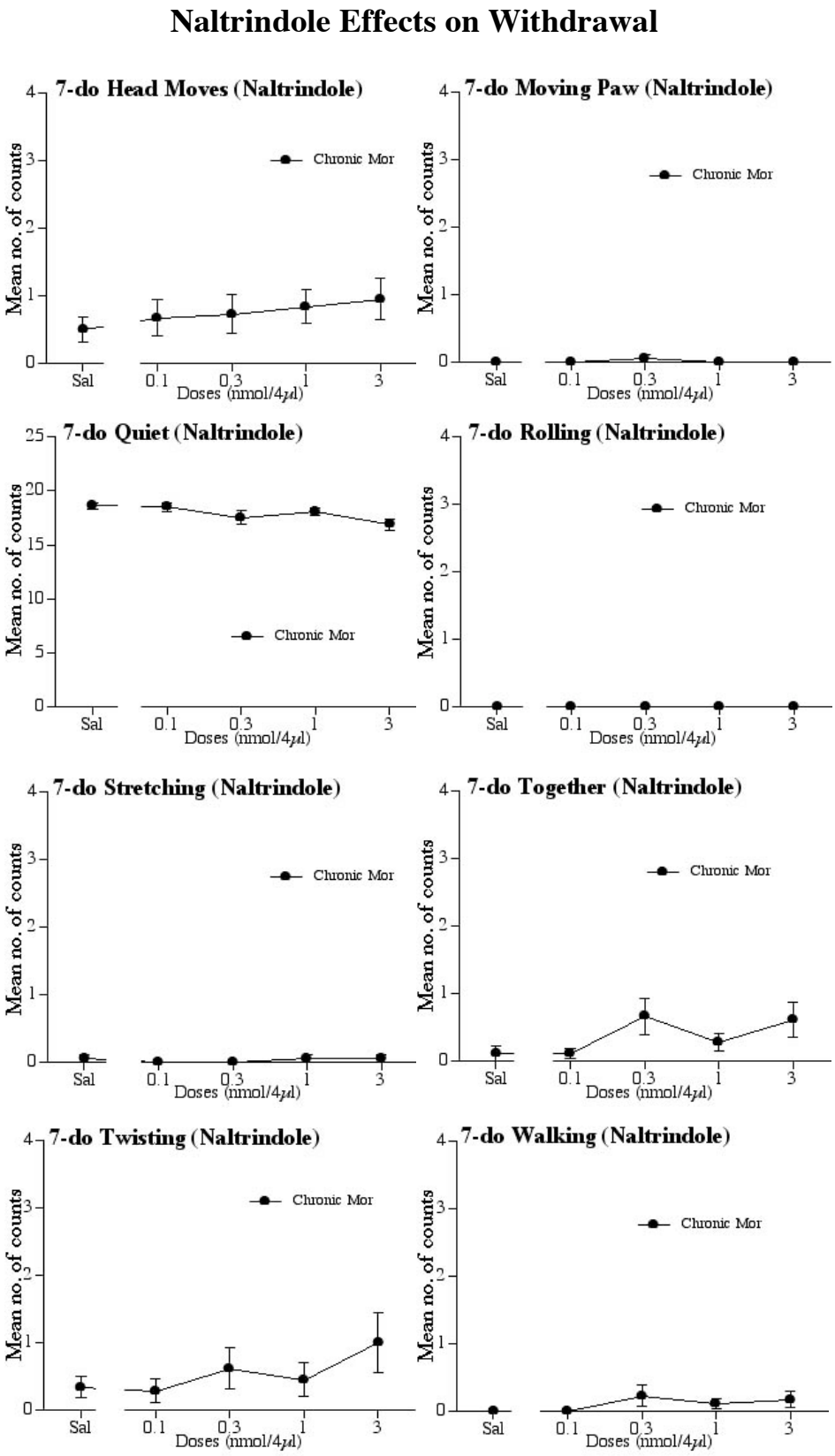


Fig 3-2. Dose effect of naltrindole, the δ-OR antagonist. This figure shows the mean number (± SEM) of occurrences, in 15 minutes, of various morphine withdrawal behaviors in the 7-day old rat. None of the behaviors showed any significant dose effects. *Quiet is presented on a different scale.

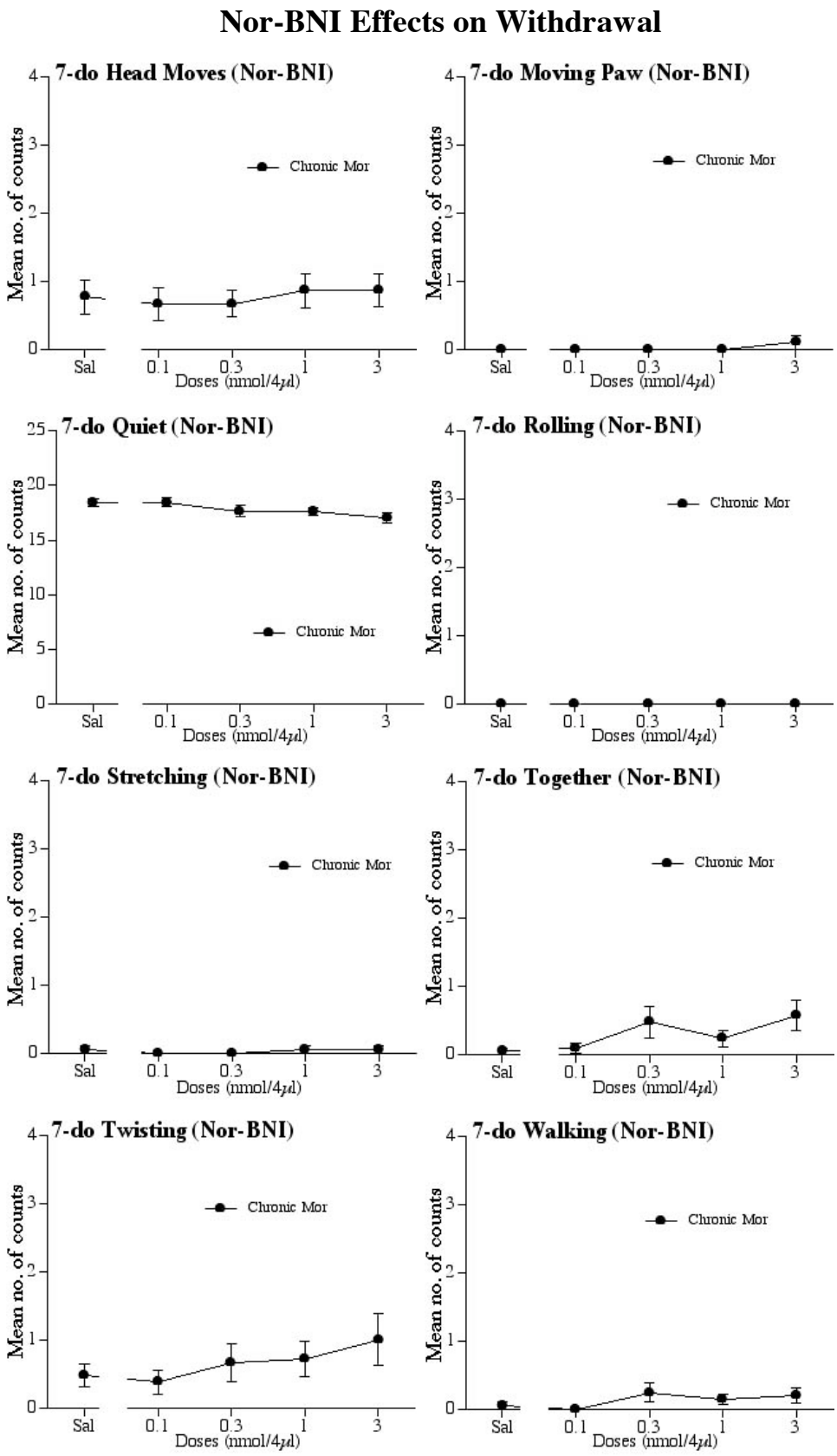


Fig. 3-3. Dose effect of nor-BNI, the κ-OR antagonist. This figure shows the mean number (± SEM) of occurrences, in 15 minutes, of various morphine withdrawal behaviors in the 7-day old rat. None of the behaviors showed any significant dose effects. *Quiet is presented on a different scale.

Chapter 4: Gene Expression During Morphine Tolerance and Dependence

Introduction

Despite their effectiveness as analgesics, prolonged exposure to opiates results in distinct biochemical and physiological changes in individual neurons and entire interacting circuits of neurons, which in turn can result in permanent alterations in cellular communication (Redmond and Krystal 1984; Koob and Bloom 1988; Nestler and Aghajanian 1997; Ingram, Vaughan et al. 1998). These neural changes form the basis of the psychological and physical dependence that is seen after chronic exposure to the opiate. As a result of these neural adaptations, removal of the opiate results in behaviors (the withdrawal syndrome) that tend to be the opposite of the initial acute effects of the drug. For instance, when human adults take morphine or heroin they experience euphoria, relaxation, hypothermia, respiratory depression, and constipation, but when withdrawing from these drugs they experience restlessness, diarrhea, psychological depression, increased blood pressure, severe muscle pains, and gastrointestinal cramps (Eddy, Halbach et al. 1965; Koob and Bloom 1988).

Numerous rodent models of opiate dependence have defined the neuroanatomical regions that are involved in the physical signs of opiate withdrawal in the adult. In an early study by Laschka et al (Laschka and Herz 1977) the injection of ^{3}H-naloxone into

the lateral ventricle and basal cisterns of morphine dependent rats, produced strong withdrawal behaviors as a result of its spread to the periaqueductal gray area (PAG), the locus coeruleus (LC), and the periventricular gray substance (PVG). More recent studies have shown that physical withdrawal can be induced by the microinjection of opiate antagonists into the amygdala, nucleus accumbens (NAcc), medial thalamus, preoptic hypothalamus, PAG, LC, and N. raphe magnus (Maldonado, Stinus et al. 1992c; Walters, Aston-Jones et al. 2000), and affective signs of withdrawal can be induced by the microinjection of antagonists into the NAcc and amygdala (Stinus, Le Moal et al. 1990; Frenois, Cador et al. 2002).

Increases in regional cerebral glucose utilization in the amygdala, hypothalamus (Adams and Wooten 1990), NAcc, thalamus, and medial and lateral preoptic areas (POA) (Wooten, DiStefano et al. 1982; Geary and Wooten 1985; Kimes and London 1989) have been used as neuronal indicators of withdrawal in the opiate dependent rodent. There are also increases in the number of cells positive for Fos-LIR in the LC (Hayward, Duman et al. 1990), NAcc (Nye and Nestler 1996; Georges, Stinus et al. 2000), N. tractus solitarius, ventrolateral medulla, raphe N., hypothalamus, amygdala (Stornetta, Norton et al. 1993; Johnstone, Brown et al. 2000; Veinante, Stoeckel et al. 2003), PAG (Chieng, Keay et al. 1995b), and Lamina I/II of the spinal cord (SC) (Rohde, Detweiler et al. 1996), as well as the levels of *c-fos* and c-jun mRNA in the LC of morphine dependent animals as a result of precipitated withdrawal (Hayward, Duman et al. 1990). When these studies are taken together, a circuit evolves for the regulation of physical dependence in the adult rodent, whose major components include (but are not limited to) the LC, PAG, amygdala, SC, hypothalamus, and NAcc.

However, little is known of the regions involved in opiate withdrawal in the infant rodent. Maeda, et al (Maeda, Kishioka et al. 2002) found dramatic increases in levels of *c-fos* mRNA in the olfactory bulb and small (but significant) increases in the hypothalamus and medulla of 7-day old morphine dependent rats experiencing naloxone-induced withdrawal. In addition, our laboratory, as well as others, have found that multiple brain regions show changes in *c-fos* mRNA (Jones, Zhu et al. 2002; Zhu, Jenab et al. 2003b) when opiate withdrawal is induced, are associated with withdrawal when microinjected with an opiate antagonist (Jones and Barr 2001), and are associated with conditioned place preference when microinjected with morphine (Barr and Rossi 1992c).

As with adults, human infants and rat pups display physical withdrawal behaviors when long-term opiate exposure is terminated. These behaviors in human infants include altered sleep patterns, high-pitched crying, respiratory and gastrointestinal dysfunction, irritability, and tremors (Finnegan 1985; Tobias, Schleien et al. 1990; Anand and Arnold 1994; Szeto 1995; Franck and Miaskowski 1998).

There are qualitative changes in physical withdrawal behaviors that are seen during human development, which are also seen throughout development in the rodent model. For instance, common behaviors seen in adult rats include wet dog shakes, teeth chattering, ptosis, pilo-erection, and jumping (Buckett 1964; Bläsig, Herz et al. 1973; Maldonado, Stinus et al. 1992c), whereas behaviors that are unique to the 7-day old rat include head movements, paw movements, twisting, stretching, rolling, and increased ultrasonic distress vocalizations (Barr and Wang 1992d; Jones and Barr 1995; Windh, Little et al. 1995b; Thornton, Wang et al. 1997). One explanation for the different behavioral and physiological changes that are seen during ontogeny may be the relative

immaturity of the neurons that comprise the individual brain loci and/or the pathways that interconnect these loci.

We hypothesized that despite the relative immaturity of the neuroanatomical loci in the 7-day old rat, areas that show activity during opiate withdrawal in the adult rat would show some level of activity in the young rat. As a marker of cellular activity the expression of the *c-fos* protein (Fos), which is rapidly stimulated in response to neuronal activity, was quantified. The presence of the protein Fos and its mRNA has been successfully used as a measure of neuronal activation throughout the CNS of the adult (Morgan, Cohen et al. 1987; Le Guen, Gestreau et al. 2001; Benavides, Laorden et al. 2003; Le Guen, Gestreau et al. 2003) and developing rodent (Williams, Evan et al. 1990; Joyce and Barr 1995; Yi and Barr 1995; Boucher, Jennings et al. 1998; Andersen, LeBlanc et al. 2001; Wiedenmayer and Barr 2001). Therefore, for the current experiment we precipitated physical withdrawal, using the opiate antagonist naltrexone, in morphine dependent 7-day old rat pups and quantified the number of Fos-like immunoreactive (Fos-LIR) cells located within the LC, NAcc, PAG, and SC.

Materials and methods

Subjects/Tattooing

As described in the general methods section.

Establishment of Tolerance

As described in the general methods section.

Withdrawal Precipitation: As described in the general methods section. Pups were injected with either saline or the broadly acting opiate antagonist naltrexone

hydrochloride (Sigma; 1mg/kg, 0.01ml/g). The presence or absence of withdrawal behaviors was noted, but not quantified.

Fixation of Tissue/Perfusion

After 2 hours, pups were sacrificed with a lethal dose of sodium pentobarbital (i.p.). This time point was chosen because it has been shown to be the time of maximal levels of the protein Fos in the nervous system (Morgan, Cohen et al. 1987) and, based on previous work in our laboratory, has been shown to be an acceptable time-point (Yi and Barr 1997; Wiedenmayer and Barr 2001). They were transcardially perfused with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The brains and spinal cords were frozen and coronally sectioned (30μm for SC and LC, 150μm for PAG and NAcc) in a cryostat. Two out of every three sections was floated in PBS, in isolated wells of a plastic container. One section was placed on a microscope slide for cresyl violet staining, to locate anatomical regions, and one was used for immunohistochemical processing.

Immunohistochemistry

Sections were processed using a modified protocol of the avidin-biotin-peroxidase (ABC) system of Hsu, et al (Hsu, Raine et al. 1981). Briefly, they were pre-incubated in 3% hydrogen peroxide for 10 min in order to quench endogenous peroxidase activity. The sections were then incubated for 48 hours at 4°C on a shaker in the primary antibody, rabbit anti-Fos (*c-fos* Ab-5; Oncogene Science, Union, NY) diluted 1:2,000 in PBS with Triton-X and 1% goat serum. They were then rinsed using PBS, incubated in the secondary antibody, goat-anti-rabbit (Vector Laboratories, Burlingame, CA) for 1 hour, and processed using the ABC kit (Vector Laboratories, Burlingame, CA). Sections were

stained using diaminobenzidine peroxidase substrate tablets (DAB; Sigma, Saint Louis, MO). Stained sections were mounted on gelatin-subbed slides, dehydrated in alcohol followed by xylene, and coverslipped. Tissue from a control pup and a treated pup were processed together and the experimenter was blind to the treatment of each pup.

Fos-LIR Quantitation

Fos-like immunoreactive (LIR) positive cells were visualized using a microscope (Nikon-Optiphot) equipped with a drawing tube (See Fig. 4-1). A person unaware of the chronic and acute treatment of the animal counted all Fos-LIR cells, regardless of the density of the staining (as long as it was distinct from the background). For each animal, the mean number of Fos-LIR cells per brain area was calculated by averaging counts from all sections.

Results

A factorial analysis of variance (ANOVA) was performed for each of the brain regions. The four treatment groups were defined as follows. 1) Experimental group: chronic morphine + acute naltrexone (M-N). 2) Control groups: chronic morphine + acute saline (M-S), chronic saline + acute naltrexone (S-N), and chronic saline + acute saline (S-S). All sections containing the brain region were counted and averaged (2-5 sections for the NAcc and SC and 4-8 sections for the PAG and LC). There were no significant differences between the left and right sides in any of the regions so the values were pooled together for analysis:

Nucleus Accumbens: There were significantly higher levels of Fos-LIR in the entire NAcc of the experimental group as compared to the controls, $F(1,9) = 9.66$, $p < 0.01$. In

addition, the shell showed higher levels of Fos-LIR as compared to the core in the experimental group, $F_{(1,8)} = 8.24$, $p < 0.05$. (Fig. 4-2)

Periaqueductal gray area: Significantly higher levels of Fos-LIR were found in the entire PAG of the experimental animals as compared to the controls, $F_{(1,8)} = 9.94$, $p < 0.05$. There were no significant differences in Fos-LIR levels between the dorsal and ventrolateral regions of the PAG. (Fig. 4-3)

Spinal Cord: There were significant increases in the levels of Fos-LIR in the experimental group as compared to the control, $F_{(1,7)} = 42.3$, $p < 0.01$. Note: these animals include only a M-S control. The changes occurred largely in Lamina IV and V (or dorsal surface) of the dorsal horn. (Fig. 4-4)

Locus Coeruleus: There were significantly higher levels of Fos-LIR in the experimental group, as compared to the controls, $F_{(1,12)} = 6.73$, $p < 0.05$. (Fig. 4-5).

Discussion

Withdrawal in the 7-day old morphine dependent rat pup, precipitated by the broadly acting opiate antagonist naltrexone was associated with increased Fos-like immunoreactivity (LIR) in several brain regions. These included the periaqueductal gray area, the nucleus accumbens, the dorsal horn of the spinal cord, and the locus coeruleus.

The increased levels of activity that were seen in the *nucleus accumbens* (NAcc), as a result of opiate withdrawal, were higher in the shell than in the core. These results are comparable to adult studies where behavioral withdrawal precipitated by the injection of opiate antagonists into the NAcc causes an increase in levels of the c-fos protein (Hayward, Duman et al. 1990; Gracy, Dankiewicz et al. 2001), *c-fos* mRNA (Hayward, Duman et al. 1990), and glucose utilization (Wooten, DiStefano et al. 1982; Geary and

Wooten 1985). Single-unit intra-accumbal recordings show that cellular activity is inhibited by the microinfusion of morphine (Hakan, Callaway et al. 1989a; Hakan and Henriksen 1989b; Chieng and Williams 1998) and extracellular levels of adenosine and its metabolites hypoxanthine and inosine are increased in the accumbens in rats undergoing opiate withdrawal (Salem and Hope 1999). In addition, the systemic administration of naltrexone causes increased Fos-related antigen immunoreactivity in chronically morphine treated rats (Walters, Aston-Jones et al. 2000) and increased CREB-mediated transcription in chronically morphine treated transgenic mice (Shaw-Lutchman, Barrot et al. 2002) in the NAcc.

The NAcc is part of the mesocorticolimbic-dopamine pathway and a critical site for the reinforcing properties of opiates (Wise 1987; Dworkin, Guerin et al. 1988; Koob, Wall et al. 1989; Shaw-Lutchman, Barrot et al. 2002). Lever pressing in morphine dependent adult rats can be significantly reduced when the opiate antagonist methylnaloxonium is microinjected into the NAcc (Koob, Wall et al. 1989; Stinus, Le Moal et al. 1990). As the major neurotransmitter responsible for the positive affects associated with this region, studies have found that chronic exposure to morphine increases dopamine's (DA) extracellular levels within the NAcc, while opiate antagonists decrease its levels (Pothos, Rada et al. 1991; Rossetti, Hmaidan et al. 1992).

In addition, chronic morphine treatment increases DA release within the core but decreases DA release within the shell (Cadoni and Di Chiara 1999). This is consistent with the current findings in which precipitated withdrawal increases levels of Fos-LIR within the shell, while the levels of Fos-LIR remain unaffected within the core. All of

which may indicate the role of the shell of the NAcc in the negative behaviors and affect associated with the use of opiates.

There is evidence that place aversions can be conditioned in rats by PD 14 (Barr and Goodwin 1997) and separation-induced ultrasonic vocalizations (USVs) (Barr and Wang 1992d) can be elicited in withdrawing rat pups younger than PD3. In addition, this laboratory has found that the opiate antagonist naltrexone injected directly into the NAcc increases USVs in 3-day old rat pups (unpublished data). Therefore the increased levels of Fos-LIR elicited in this current study may represent cellular mechanisms of affective withdrawal as evidenced by increased ultrasonic vocalizations from the previous study.

In addition to the NAcc, increases in levels of Fos-LIR were seen in the *periaqueductal gray area* (PAG) of the 7-day old rat, although no differences were found between its dorsal and ventrolateral subregions. Previously, Jones (Jones and Barr 2001) elicited withdrawal behaviors similar to those seen after the systemic injection of naltrexone, by the direct injection of methylnaloxonium into the ventral PAG of the same age group. When combined with our current data, there is strong evidence that the PAG of the 7-day old rat becomes increasingly active during opiate withdrawal, as is the case with the adult rat.

The adult rodent contains a large number of opioid receptors in the PAG (Vaughan and Christie 1997; McNally, Pigg et al. 2004) and is therefore sensitive to the administration of opiate antagonists and agonists. Physical dependence can be induced by the chronic administration of enkephalin analogues into the PAG (Maldonado, Fournie-Zaluski et al. 1992a), and a pronounced withdrawal syndrome is seen following the administration of various opiate antagonists (Laschka, Teschemacher et al. 1976b; Laschka and Herz 1977;

Aghajanian 1978; Maldonado, Fournie-Zaluski et al. 1992a). Physical withdrawal can be attenuated by the direct administration of enkephalin catabolism inhibitors into the PAG (Maldonado, Fournie-Zaluski et al. 1992a). At the cellular level, increases in metabolic activity are seen during withdrawal (Kimes and London 1989) as well as increases in Fos-LIR in the neurons of the ventrolateral PAG (Chieng, Keay et al. 1995b). In addition, the intra-ventrolateral injection of the opiate antagonist naloxone blocked fear conditioning in rats (McNally, Pigg et al. 2004). Worth noting is that the caudal portion of the PAG is in close proximity to the LC leading some researchers to suggest that much of the activity following opiate administration or precipitated withdrawal, that is attributed to neurons of the LC is in fact the result of the activity of neurons within the PAG (Christie, Williams et al. 1997).

Another locus showing significant increases in levels of Fos-LIR upon the precipitation of morphine withdrawal in the 7-day old rat is the *spinal cord* (SC), specifically the dorsal horn. As the initial CNS structure to receive afferent information, the spinal cord plays a regulatory role in the opiate system primarily through its modulation of analgesia. Moreover, it has also been shown to play a significant role in opiate dependence and physical withdrawal in the adult rodent. For instance, chronic intrathecal (IT) injections of morphine increase levels of PKC-dependent phosphorylation, as well as PKC-α and PKC-γ immunoreactivity in the dorsal horn of the SC (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000). Acute exposure to morphine blocks calcium entry into the SC (Bernstein and Welch 1995), resulting in a decreased production of cAMP. Pretreatment with an IT injection of an opiate antagonist blocks the development of dependence (Delander and Takemori 1983) and the

precipitation of withdrawal increases levels of Fos-LIR within the dorsal horn of the SC (Rohde, Detweiler et al. 1996).

In the infant rat, there is evidence for the role of the spinal cord in opiate tolerance and withdrawal. In an isolated spinal cord preparation from 7-, 14-, and 21-day old rats that had been chronically treated with morphine, the addition of naltrexone to the bath increased the magnitude of the electrically evoked sVRP's (Zhu 2002). The SC contains the greatest concentration of opioid receptors in the dorsal horn and therefore our results from this current study were exactly as expected given the characteristics of the SC.

The final brain structure where levels of Fos-LIR were significantly increased during precipitated withdrawal was the *locus coeruleus* (LC). The LC is the major site of norepinephrine (Leshner 1996), within the CNS, and contains a high density of opioid receptors (Przewlocka, Turchan et al. 1996; Van Bockstaele, Colago et al. 1996). It has been shown to be involved in learning and memory, anxiety, pain, and attention, as well as autonomic signs of withdrawal (Stornetta, Norton et al. 1993; Sim-Selley, Selley et al. 2000). In addition, the LC is functional by PD7 (Wilson and Leon 1988) and the intra-LC injection of an opiate antagonist elicits withdrawal in morphine dependent 7-day old rats (Jones and Barr 2001).

Behavioral research has shown that microinjections of opiate antagonists into the LC of morphine dependent adult rats (Koob, Maldonado et al. 1992; Maldonado, Stinus et al. 1992c) causes withdrawal signs and the lesioning of the LC results in reduced withdrawal signs (Maldonado and Koob 1993). However, the vast amount of published research on the LC has focused on it as a model for the long-term neuronal changes resulting from opiate exposure. For instance, acute administration of opiates inhibits the firing rate of

LC neurons (Korf, Bunney et al. 1974; Sharma, Klee et al. 1975; Bird and Kuhar 1977; Hayward, Duman et al. 1990) and reduces levels of cAMP and cAMP-dependent protein kinases (PKA) (Guitart and Nestler 1993). Zhu and Zhou (Zhu and Zhou 2001c) found that the reduced firing rate resulted in long lasting synchronous discharges within a sub-population of LC neurons, which in turn may be responsible for the synaptic plasticity that is the cellular basis of opiate dependence (Aghajanian 1978; Foote, Bloom et al. 1983).

Chronic administration of opiates causes the firing rates to return to baseline as the neurons develop tolerance to the drug (Sharma, Klee et al. 1975; Andrade, Vandermaelen et al. 1983). Finally, during precipitated withdrawal increased activity of LC neurons has been observed (Sharma, Klee et al. 1975; Hayward, Duman et al. 1990; Maldonado, Stinus et al. 1992c; Guitart and Nestler 1993; Aston-Jones, Hirata et al. 1997) as well as enhanced metabolic activity (Kimes and London 1989; Maldonado, Stinus et al. 1992c).

Although controversy has surrounded the extent to which the LC modulates opiate dependence (Andrade, Vandermaelen et al. 1983; Christie, Williams et al. 1997,Chieng, 1995 #444), it is still apparent that the LC becomes active during opiate withdrawal in the adult rodent. Adding to the extensive body of published research on the adult rodent is our research (as well as that of others) on the infant rodent, where activity in the LC is observed during opiate withdrawal.

Despite the fact that decades of research have gone into understanding these underlying mechanisms, little is still known about infants and opiate dependence. Are the events that unfold in the infant rodent (and human) during chronic exposure to opiates qualitatively similar, somewhere in between, or completely different from those seen in adult rodents?

We hypothesized that, although not as dramatic as that seen in the adult rat, the morphine dependent infant rat would show increased cellular activity (as measured by increases in levels of Fos-LIR) as a result of precipitated withdrawal in the LC, NAcc, PAG, and SC, results similar to those found in the adult literature. In all areas studied, we found evidence to support our hypothesis. The exact role that each region plays in opiate dependence and withdrawal will require further investigation.

Photomicrograph of The Periaqueductal Gray Area

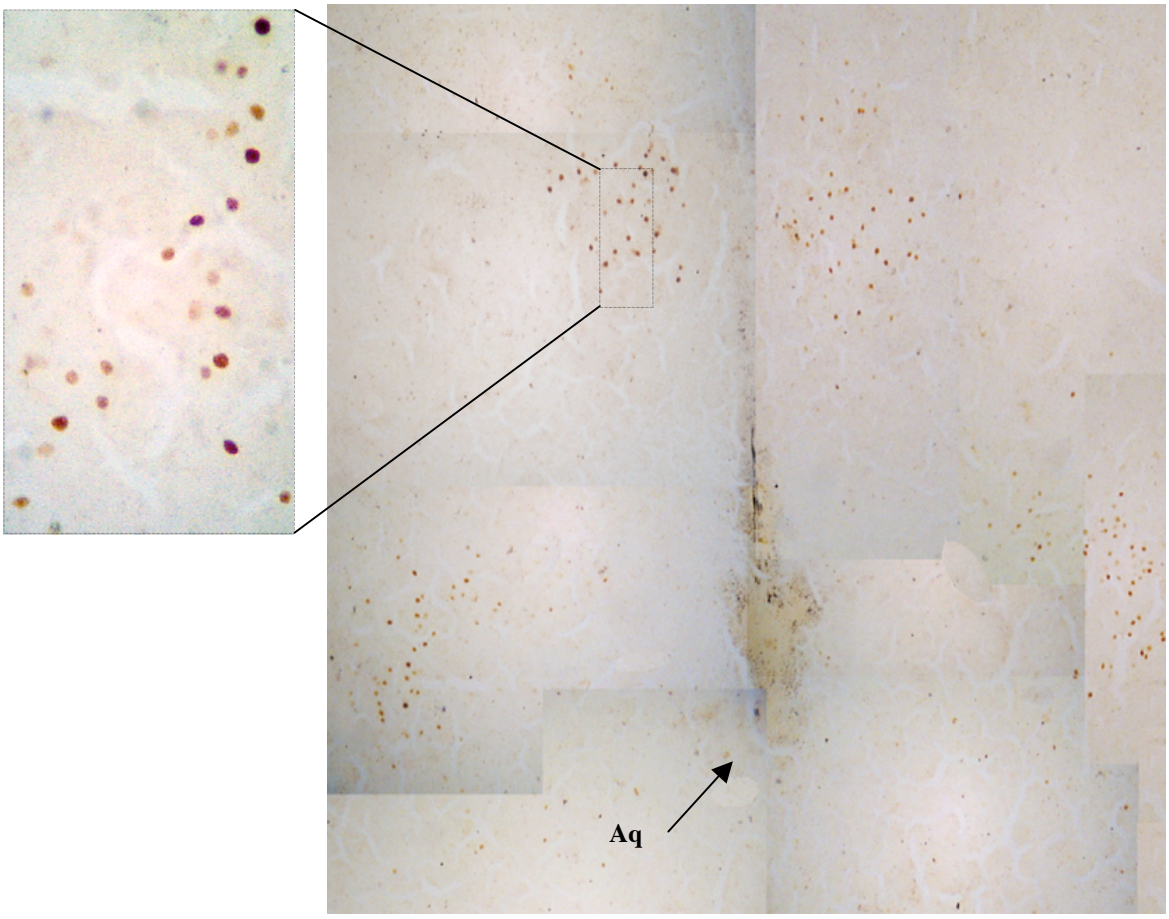


Fig 4-1. Photomicrograph showing the distribution of Fos-like immunoreactive cells in a coronal section of the periaqueductal gray area. The animals were treated chronically (7 days) with morphine and given an acute injection of naltrexone to precipitate withdrawal. The panel to the left is a higher magnification of the same section. "Aq" indicates the cerebral aqueduct.

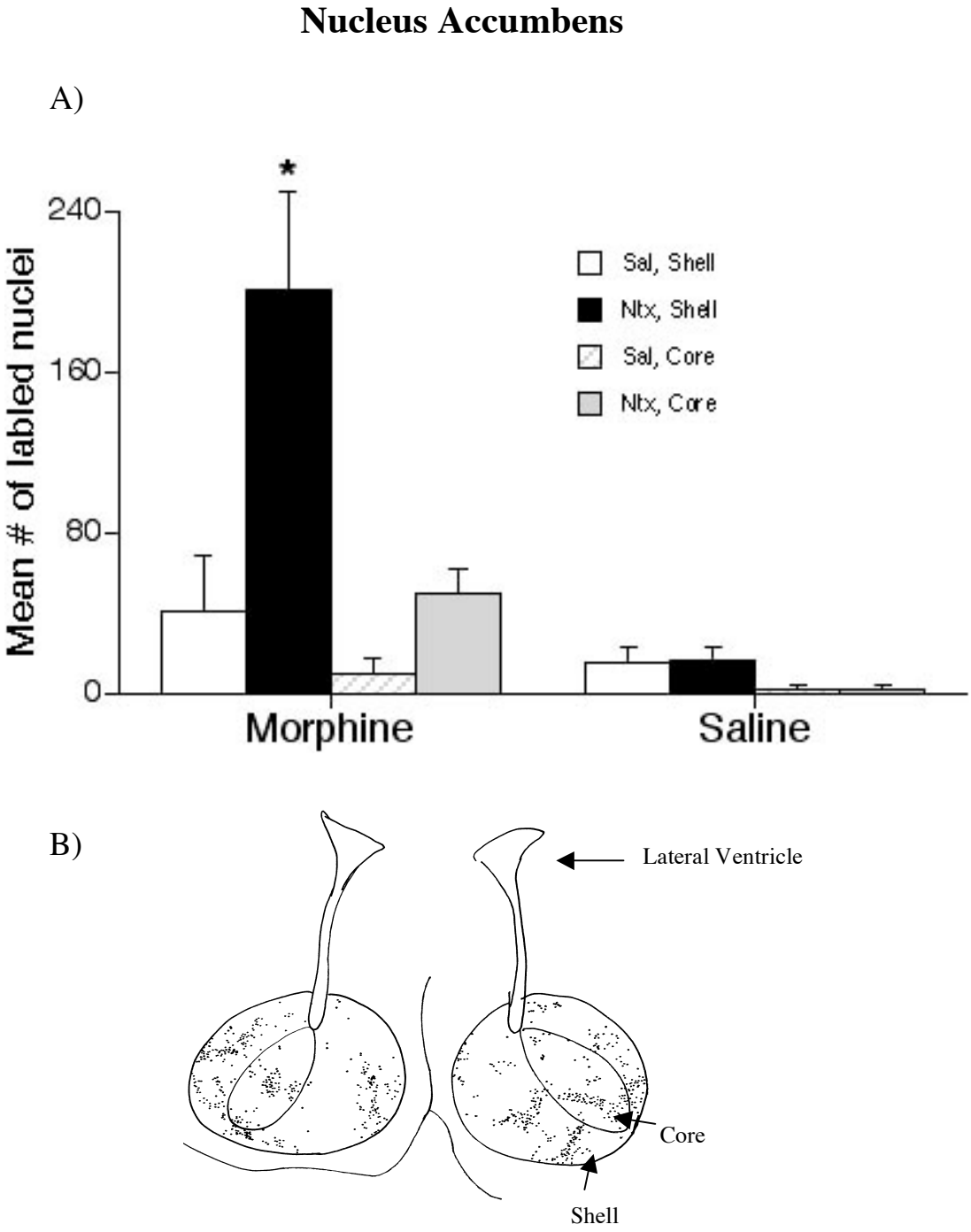
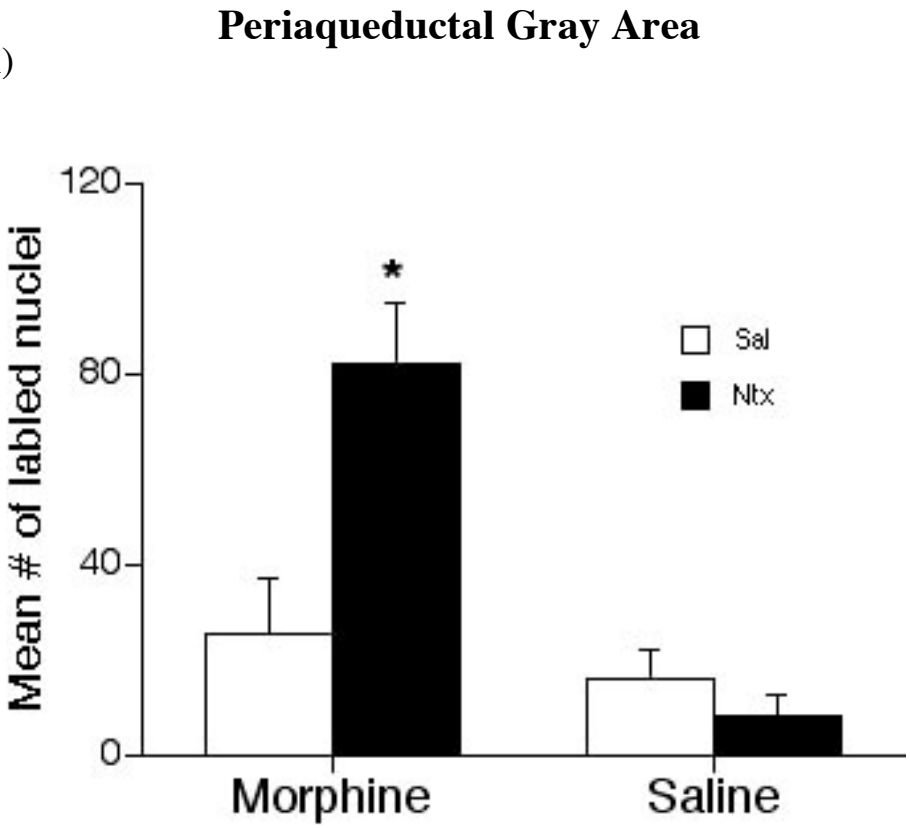


Fig. 4-2. A) Mean number of Fos-like immunoreactive cells in the nucleus accumbens of 7-day old rats that were chronically exposed to morphine/saline and then given an acute injection of naltrexone/saline. (N = 5 rats chronically treated with morphine, 5 rats chronically treated with saline; mean ± S.E.) Asterisk (*) denotes significance between that group and all controls. (B) Camera lucida drawing of the shell and core of the nucleus accumbens. The animals were treated chronically with morphine and given an acute injection of naltrexone to precipitate withdrawal.

A)



B)

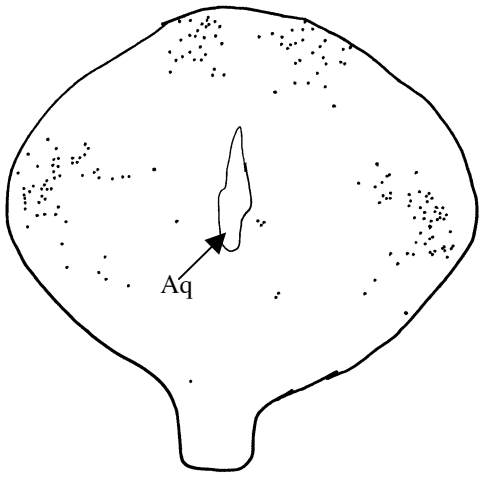
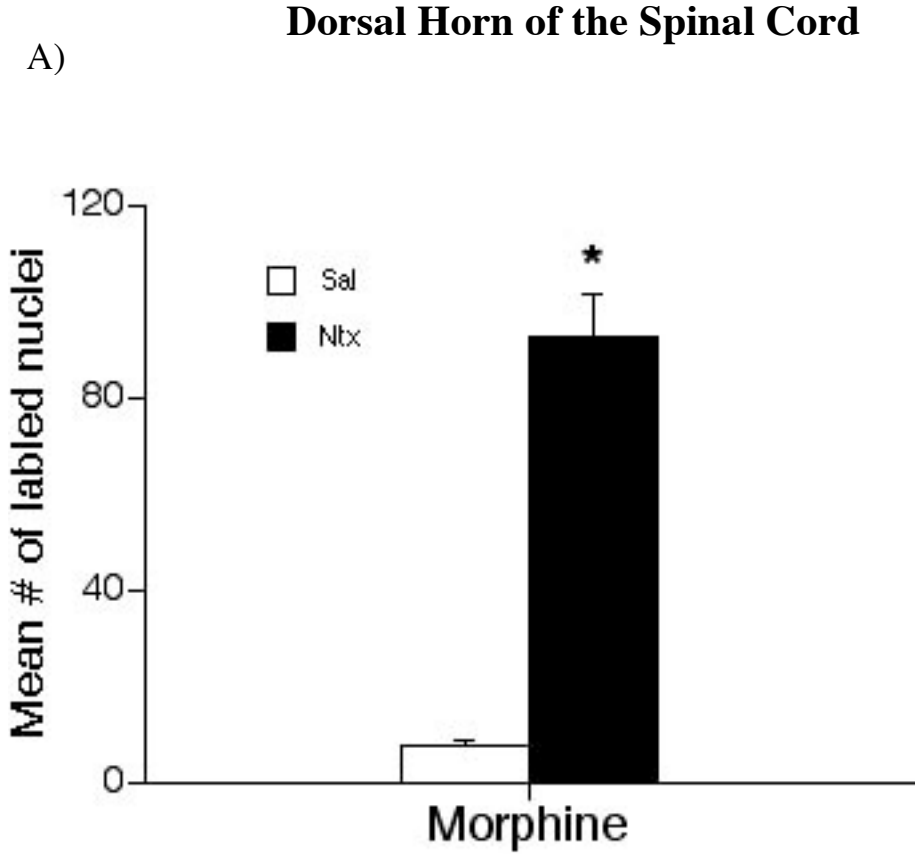


Fig. 4-3. (A) Mean number of Fos-like immunoreactive cells in the periaqueductal gray area of 7-day old rats that were chronically exposed to morphine/saline and then given an acute injection of naltrexone/saline. (N = 5 rats chronically treated with morphine, 5 rats chronically treated with saline; mean ± S.E.). Asterisk (*) denotes significance between that group and all controls. (B) Camera lucida drawing of the periaqueductal gray area. The animals were treated chronically with morphine and given an acute injection of naltrexone to precipitate withdrawal.

A)



B)

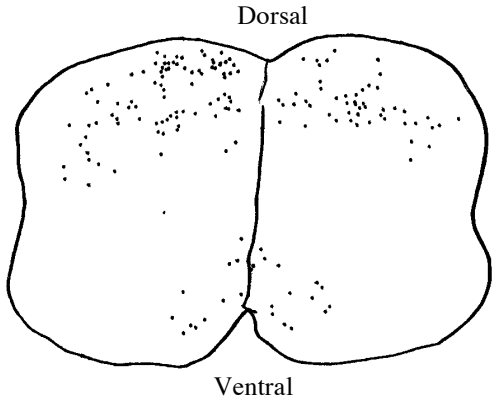
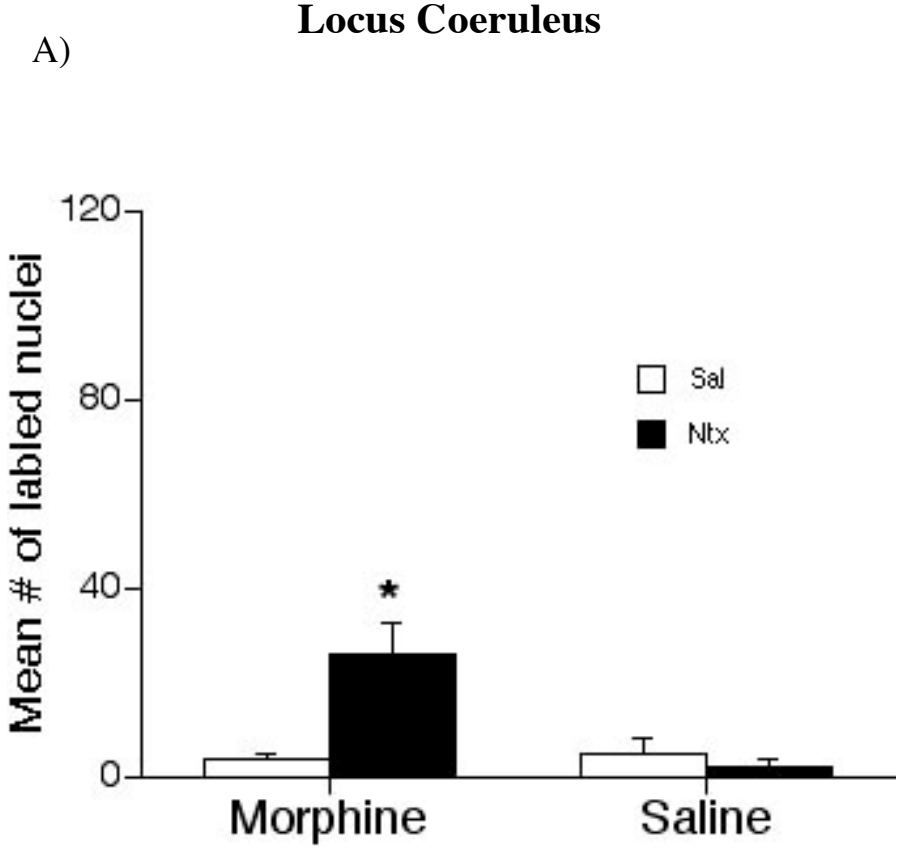


Fig. 4-4. (A) Mean number of Fos-like immunoreactive cells in the dorsal horn of the spinal cord of 7-day old rats that were chronically exposed to morphine and then given an acute injection of naltrexone/saline. Control animals show little or no Fos expression and thus are not shown here. (N = 8 rats chronically treated with morphine; mean ± S.E.). Asterisk (*) denotes significance between that group and controls. (B) Camera lucida drawing of the spinal cord. More label cells can be seen in the dorsal region of the spinal cord. The animals were treated chronically with morphine and given an acute injection of naltrexone to precipitate withdrawal.

A)



B)

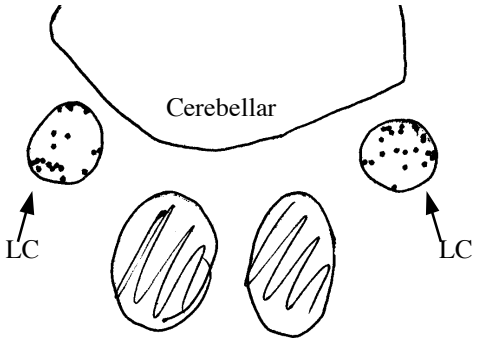


Fig. 4-5. (A) Mean number of Fos-like immunoreactive cells in the locus coeruleus of 7-day old rats that were chronically exposed to morphine/saline and then given an acute injection of naltrexone/saline. (N = 8 rats chronically treated with morphine, 6 rats chronically treated with saline; mean ± S.E.). Asterisk (*) denotes significance between that group and all controls. (B) Camera lucida drawing of the locus coeruleus. The animals were treated chronically with morphine and given an acute injection of naltrexone to precipitate withdrawal.

Part Six: The Protein Kinase and Opiate Interaction – Part 2 of Young Rat Experiments

Explanation

The next step, after chapters 3 and 4 was to bring everything (receptors and genes) together by focusing on the protein kinases. Which are proteins that have been shown to play a crucial role in opiate tolerance and dependence through a wide variety of experimental paradigms.

Chapter 5

Protein kinases (PK) are a class of enzymes that phosphorylate proteins, causing a conformational change that results in altered functioning of that target protein. This function of PKs is hypothesized to be a crucial step in the signal transduction cascade and has resulted in numerous behavioral pharmacological studies describing the effects of PK inhibitors and activators on the expression of physical opiate tolerance and dependence. The set of experiments described in this chapter were designed to give insight into the effects of PK activity on opiate tolerance and dependence in the young rat. The central question being addressed was, will blocking the activity of the protein kinases, PKC and PKA effect the expression of behavioral tolerance and dependence of rats pups chronically exposed to morphine? The results of this chapter were presented at the annual meetings of the International Society for Developmental Psychobiology (2001) and the Society for Neuroscience (2003 and 2004).

Chapter 6

If the opiate system is truly regulated by protein kinases, it is theoretically possible that the two systems are so intertwined that the protein kinases can then be regulated by opiates. This of course is the case and the bases for many of the earlier studies that

sought to understand the underlying mechanisms of opiate tolerance and dependence. In a classic study done by Sharma et al (Sharma, Klee et al. 1975) it was hypothesized that acute morphine treatment results in decreased adenylyl cyclase activity and decreased cAMP levels. Long-term exposure brings activity and levels back to control (representing the biochemical equivalence of tolerance and dependence) and acute administration of an opiate antagonist causes adenylyl cyclase activity and cAMP levels to "shoot" up beyond control levels (representing a biochemical equivalence of withdrawal) (Nestler and Tallman 1988). See figure 2-3. Therefore, the experiments in this chapter were designed to determine if opiates are able to regulate PK levels in the young rodent. The findings of this chapter were presented at the annual meeting of the Society for Neuroscience (2004).

Chapter 5: The Modulation of Morphine Tolerance and Dependence by Protein Kinases (Behavioral Analysis)

Introduction

The behavioral effects of long-term exposure to opiates have been well documented in the human literature and more recently in rodent models. These effects include tolerance to the opiate, such that more of the opiate is needed to produce the same effect. Dependence to the drug also occurs and is indicated by the occurrence of withdrawal behaviors upon removal of the opiate.

In an attempt to further understand tolerance and dependence, and determine pharmacological substances to reduce or eliminate their symptoms, a large body of experimental studies has focused on the underlying molecular mechanisms that modulate their development and expression. Of particular interest are protein kinases because of their post-translational modifications, such as the ability to be phosphorylated and phosphorylate other proteins. Protein phosphorylation alters the activity of the target protein and this process is thought to be crucial in the cellular adaptations that occur as a result of long-term opiate exposure (Tokuyama, Feng et al. 1995a; Narita, Mizoguchi et al. 1996; Cerezo, Laorden et al. 2002; Smith, Javed et al. 2002).

Protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) are major participants in the IP_3-DAG and cAMP/adenylyl cyclase signal transduction pathways, respectively, and have been shown to play an important regulatory role in tolerance and dependence. The acute spinal (Bernstein and Welch 1997) and supraspinal (Bernstein and Welch 1997; Smith, Javed et al. 2003; Javed, Dewey et al. 2004) administration of various PKA antagonists reverses the appearance of the tolerance and withdrawal behaviors (Punch, Self et al. 1997) normally seen with chronic opiate treatment. The chronic co-administration of a PKC antagonist with various opiate antagonists blocks withdrawal (Aley and Levine 1997b; Cerezo, Laorden et al. 2002) and reverses tolerance (Smith, Lohmann et al. 1999b; Granados-Soto, Kalcheva et al. 2000; Smith, Javed et al. 2003), but the acute administration fails to block tolerance (Aley and Levine 1997b; Granados-Soto, Kalcheva et al. 2000) or withdrawal (Fundytus and Coderre 1996). Finally, tolerance can be blocked by the chronic co-administration of broadly acting protein kinase antagonists and opiates (Narita, Feng et al. 1994a), while withdrawal can be attenuated by the chronic co-administration (Tokuyama, Feng et al. 1995a; Tokuyama, Ho et al. 2000) or an acute injection (Maldonado, Valverde et al. 1995) of a broadly acting protein kinase antagonist.

Currently, there is very little published data on the role of PKA and PKC on opiate tolerance and dependence during the ontogeny of the rat. Therefore the following set of experiments was designed to determine the effects of PKA and PKC activity on tolerance and dependence in the young rodent. Rat pups were made dependent on morphine and on postnatal day (PD)7 or 21 they were given an acute injection of a broadly acting protein kinase antagonist, a specific PKC antagonist, or a specific PKA antagonist and then

tested for tolerance or dependence. It was hypothesized that at PD21 tolerance and dependence would be decreased by the acute administration of a protein kinase antagonist, as seen in the adult rodent, but at PD7 the antagonists would have little to no effect on the expression of tolerance and withdrawal behaviors.

Materials and Methods

Subjects/Tattooing

As described in the general methods section.

Induction of Chronic Morphine Exposure.

As described in the general methods section.

Blockade of Protein Kinases.

Two hours after the final morning injection, general protein kinase activity was blocked with one of 4 doses of the broadly acting protein kinase antagonist 1-(5-Isoquinolinylsulfonyl)-2-mehtylpiperazine dihydrochloride (H-7; Sigma; 3-, 10-, 30-, or 100nmol/4μl), PKC activity was blocked by one of 3 doses of the PKC antagonist Chelerythrine (Calbiochem; 0.1-, 0.3-, and 1nmol/4μl), and PKA was blocked by one of 3 doses of the PKA antagonist KT5720 (Calbiochem; 0.3-, 1-, and 3nmol/4μl). All drugs were injected i.c.v. as described in (Barr, Miya et al. 1992b). After the injection, behavioral testing was begun for each pup one hour (H-7) or ten minutes (Chelerythrine and KT5720) after it was returned to its littermates.

Precipitation of Withdrawal

One hour after the injection of H-7, withdrawal was precipitated with naltrexone (i.p., 1mg/kg) as described in the general methods section.

Withdrawal Behavioral Assessment

Ten minutes after being returned to their tubs, the withdrawal behavior of the target pup was observed and quantified as described in the general methods section.

Tolerance Behavioral Assessment

One hour or ten minutes after the H-7 or Chelerythrine and KT5720 injections, respectively, the tail-flick test was performed on the target pup as described in the general methods section.

Results

Protein Kinase Blockade of Opiate Withdrawal

A one-way analysis of variance (ANOVA) was used to determine the effect of the acute administration of the broadly acting protein kinase antagonist H-7 on opiate withdrawal. For both ages the treatment group was chronic morphine + acute naltrexone (MN) and the control groups were chronic morphine + acute saline (MS), chronic saline + acute naltrexone (SN), and chronic saline + acute saline (SS). Chronic morphine treatment was the between group measurement and acute H-7 and naltrexone treatment were both within measurements. The two ages were not compared in the analyses. For each behavior in both age groups the sample size was 10. *PD7*: There was no significant reduction of any of the withdrawal behaviors as a result of the acute administration of H-7. See figure 5-1; table 5-1. *PD21*: There was no significant reduction of the expression of withdrawal behaviors as a result of the acute administration of H-7. See figure 5-2; table 5-1. However, the majority of the behaviors at both ages showed significant increases when chronic treatment and acute treatment were compared in the ANOVA (data not shown). Therefore, an additional ANOVA was performed with the MN groups only, but no further significance was found. See figures 5-3 and 5-4; table 5-2. To

further assess whether treatment effects were different from controls the means from the MN groups and all control groups (collapsed into one "sal" group) were plotted as confidence intervals at the 95% level. See figures 5-5 and 5-6; table 5-3. Results from the analysis showed that at PD7 all behaviors were out of the range of the "sal" group, while at PD21 the behaviors (with the exception of head moves) were within or close to the confidence interval.

Protein Kinase Blockade of Opiate Tolerance

Mean latency values were plotted to determine the effects of the acute administration of the broadly acting protein kinase antagonist H-7, the specific PKC antagonist chelerythrine, and the specific PKA antagonist KT5720 on opiate tolerance. For both ages the treatment group was chronic morphine + acute protein kinase antagonist and the control group was chronic saline + acute protein kinase antagonist. For both age groups the sample size was 8. *PD7:* The acute administration of neither H-7, chelerythrine, nor KT5720 attenuated the degree to which this age group expressed analgesic tolerance. See figure 5-7; table 5-4. *PD21:* As with the 7-day old pups, the acute administration of neither H-7, chelerythrine, nor KT5720 attenuated the degree to which this older age group expressed tolerance. See figure 5-8; table 5-5.

Discussion

The goal of the experiments described in this chapter was to determine if protein kinases play a modulatory role in the expression of physical opiate tolerance and dependence during the early ontogeny of the rat. To determine this, animals were exposed to morphine for 6.5 days, from PD1-7 or PD15-21. On the 7th day, one set of pups in a litter was given an acute injection of the broadly acting protein kinase

antagonist H-7, followed by an injection of naltrexone to block morphine activity, and observed for the expression of withdrawal behaviors. Another set of pups was injected with H-7, the specific PKC antagonist chelerythrine, or the specific PKA antagonist KT5720, followed by 4 challenge doses of morphine and tested for the expression of analgesic tolerance using the warm water tail-flick test.

Protein Kinase Blockade of Opiate Withdrawal

Animals tested on PD7 showed no blockade of morphine withdrawal behaviors at any of the doses of H-7. See figures 5-1, 5-3 and 5-4; tables 5-1, 5-2, and 5-3. Although there were no significant effects of acute treatment with H-7 on the withdrawal behaviors seen at PD21 either, the dose-response curves showed some movement back toward control levels of all of the behaviors with the exception of head moves. See figures 5-2, 5-4 and 5-6; tables 5-1, 5-2, and 5-3.

Going on the assumption that the CNS of the 7-day old rat pup is less developed than that of the adult rat, the current findings for this age were exactly what was expected. For instance, adenylyl cyclase is detectable and shows low levels of activity by PD1 (Ihnatovych, Novotny et al. 2002b), cAMP is detectable by embryonic day 14.5 (Rius, Mollner et al. 1994), and PKC is detectable by PD7 (Hashimoto, Ase et al. 1988; Herms, Zurmohle et al. 1993; Jiang, Naik et al. 1994). Although each molecule is present, adenylyl cyclase is the only one that shows some level of activity; which might explain why there was no effect at PD7, since the protein kinases may not yet be functional at this age. In addition, Zhu and Barr (2000; 2001) were unable to block opiate withdrawal behaviors by either the chronic co-administration of an NMDA receptor antagonist with morphine or acute treatment of the NMDA receptor antagonist on the day of testing at

PD7. In fact, the expression of some of the behaviors was increased by the acute administration of the NMDA receptor antagonist, further evidence of altered functioning at this age.

Zhu and Barr (2001) also found that by PD14 co-administration of the NMDA receptor antagonist with morphine blocked the development of withdrawal, just as it does in adult rats. As a result, they hypothesized that there is a transition period around the second week of life, during which the NMDA receptor becomes functionally involved in opiate dependence. Therefore, the lack of effect from acute H-7 treatment for the 21-day old animals was unexpected.

The slight reduction of burrow, grooming, and walking may be evidence of the beginning of an effect from the acute administration of H-7. Perhaps to see a stronger effect more ages should have been tested, although it is unlikely that PKA and PKC are still non-functional by PD21 because they play such an integral role in normal cellular activity. The more likely explanation is the treatment paradigm. By giving an acute injection of H-7, only the *expression* of withdrawal was tested, but if the animals had been co-administered with H-7 and morphine the *development* of dependence would have been tested and there may have been a more robust effect (Aley and Levine 1997b; Smith, Javed et al. 2002). In fact, there is evidence that the chronic co-administration of morphine with H-7 (Tokuyama, Ho et al. 2000) or chelerythrine (Fundytus and Coderre 1996) blocks withdrawal behaviors, but the acute administration of chelerythrine does not (Fundytus and Coderre 1996). In another study, morphine withdrawal was unsuccessfully blocked by the acute administration of two different PKC antagonists, because withdrawal was still observed 30 minutes and 24 hours later (Smith, Javed et al.

2002). A number of studies, however, have been successful at blocking opiate withdrawal with the acute administration of H-7 (Maldonado, Valverde et al. 1995) and the PKA inhibitor RP-cAMPS (Punch, Self et al. 1997), although they injected the antagonists directly into the PAG and LC. These all suggest that a longer exposure time to the protein kinase antagonists or direct administration to a brain site may be needed to see a strong effect in the young rodent.

Protein Kinase Blockade of Opiate Tolerance

Opiate tolerance was not attenuated by acute doses of H-7, chelerythrine, or KT5720 at PD7 (figure 5-7, table 5-4) or PD21 (figure 5-8, table 5-5). As with withdrawal, because of the relative immaturity of the 7-day old CNS none of the antagonists were expected to have an effect on tolerance. However, the fact that they had no effect at PD21 was very unexpected. Once again, it may be a result of the specific paradigm used for the current experiments. Other developmental studies done in our laboratory show that opiate tolerance is also not attenuated at PD7 by the administration of an NMDA receptor antagonist, but tolerance is attenuated at later developmental stages such as at PD14 and 21 (Zhu and Barr 2003a). Taken alone it is possible to believe that the differences are due to the fact that this study chronically co-administered the antagonist with morphine. However, literature on the adult rodent consistently shows attenuation of tolerance, regardless of the paradigm used.

The acute administration of a PKA (Bernstein and Welch 1997; Smith, Javed et al. 2003; Javed, Dewey et al. 2004) or PKC (Granados-Soto, Kalcheva et al. 2000; Smith, Javed et al. 2003; Javed, Dewey et al. 2004) antagonist to chronically morphine treated animals successfully attenuates tolerance. The chronic co-administration of H-7 (Narita,

Feng et al. 1994a) or a PKC antagonist (Granados-Soto, Kalcheva et al. 2000) with morphine also attenuates analgesic tolerance. In addition, a PKCγ mutant mouse shows a reduction in tolerance on the tail-flick test, as compared to the wild type (Zeitz, Malmberg et al. 2001).

The overall findings of the experiments described in this chapter are inconclusive at this time. The lack of blockade of opiate withdrawal and opiate tolerance at both age groups by H-7, chelerythrine, and KT5720 may be an indicator that PKA and PKC do not play a regulatory role before PD21. If this is the case, it is in stark contrast to a large body of adult literature and developmental work done in our laboratory. On the other hand, the lack of effect may be the result of a paradigm that did not produce results that were robust enough to be identified. The latter point is the likely explanation, indicating that no true conclusion can be drawn about the modulatory role of PKA and PKC on opiate tolerance and dependence during ontogeny until additional, modified experiments have been performed. It is therefore necessary to utilize multiple paradigms in assessing PKA and PKC effects on opiate tolerance and dependence.

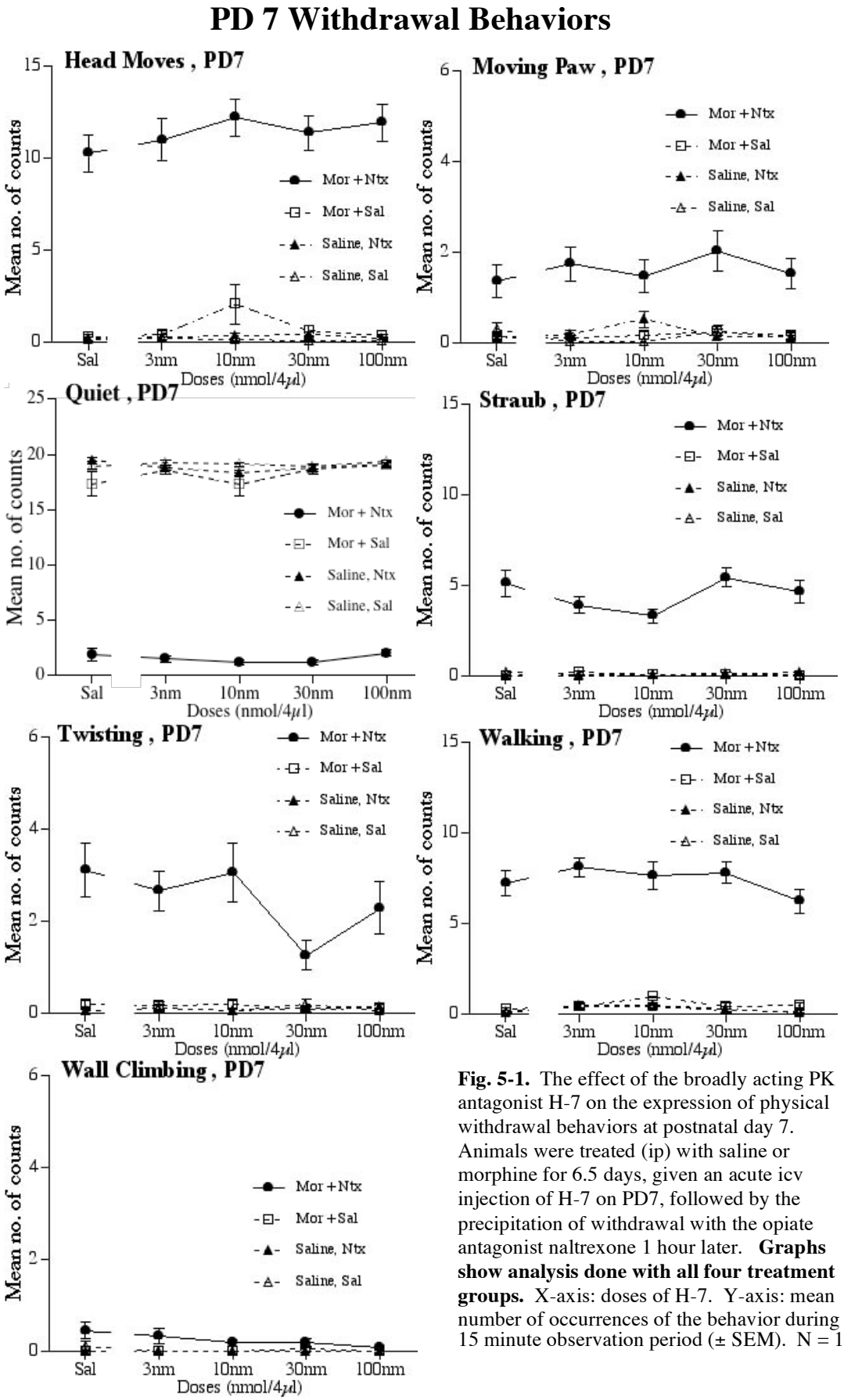


Fig. 5-1. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 7. Animals were treated (ip) with saline or morphine for 6.5 days, given an acute icv injection of H-7 on PD7, followed by the precipitation of withdrawal with the opiate antagonist naltrexone 1 hour later. **Graphs show analysis done with all four treatment groups.** X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

PD 21 Withdrawal Behaviors

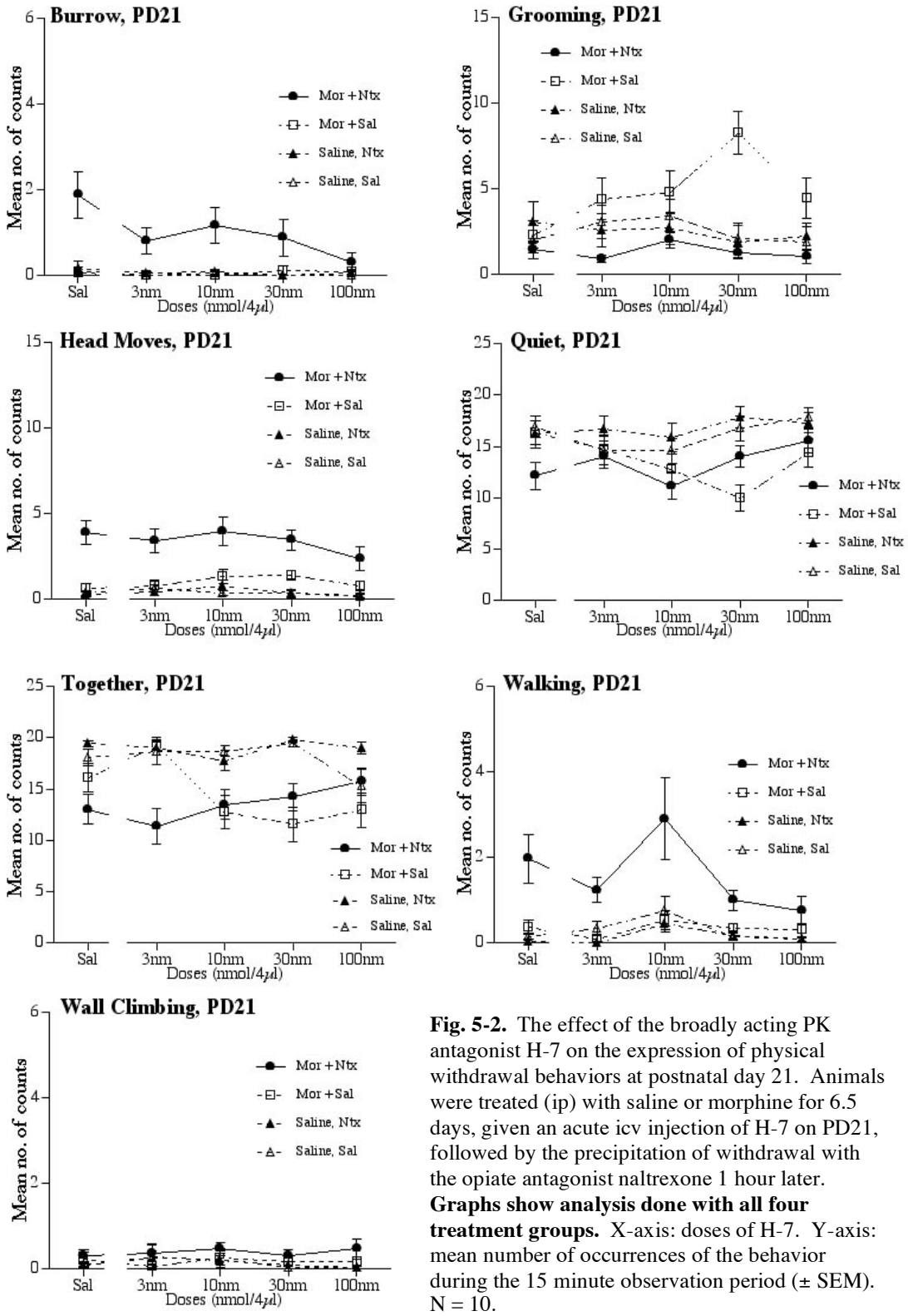


Fig. 5-2. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 21. Animals were treated (ip) with saline or morphine for 6.5 days, given an acute icv injection of H-7 on PD21, followed by the precipitation of withdrawal with the opiate antagonist naltrexone 1 hour later. **Graphs show analysis done with all four treatment groups.** X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

Withdrawal Behavior Statistics

7do

Behavior	F	df	P
Head Moves	0.25	(4,140)	0.91
Moving Paw	1.0	(4,140)	0.41
Quiet	0.28	(4,140)	0.89
Straub	1.58	(4,140)	0.18
Twisting	0.79	(4,140)	0.53
Walking	0.65	(4,140)	0.63
Wall Climbing	2.04	(4,140)	0.09

21do

Behavior	F	df	P
Burrow	0.75	(4,132)	0.56
Grooming	0.61	(4,132)	0.65
Head Moves	0.3	(4,132)	0.87
Quiet	0.66	(4,132)	0.62
Together	2.0	(4,132)	0.10
Walking	0.73	(4,132)	0.57
Wall Climbing	0.91	(4,132)	0.99

Table 5-1. Statistical analysis of the effects of H-7 on the expression of physical withdrawal behaviors a both age groups. **Analysis was done using all four treatment groups.** No significance was found.

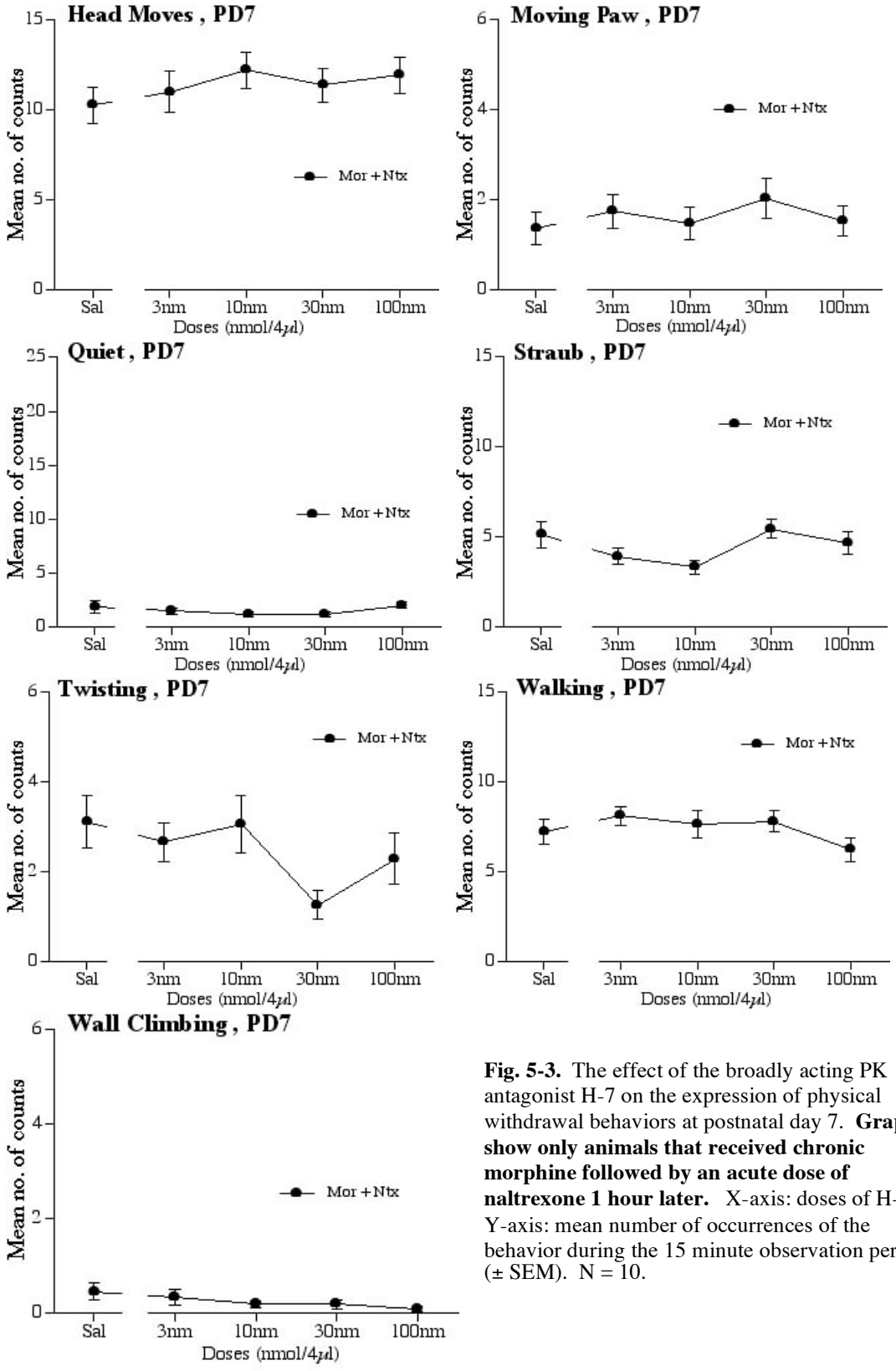


Fig. 5-3. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 7. **Graphs show only animals that received chronic morphine followed by an acute dose of naltrexone 1 hour later.** X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

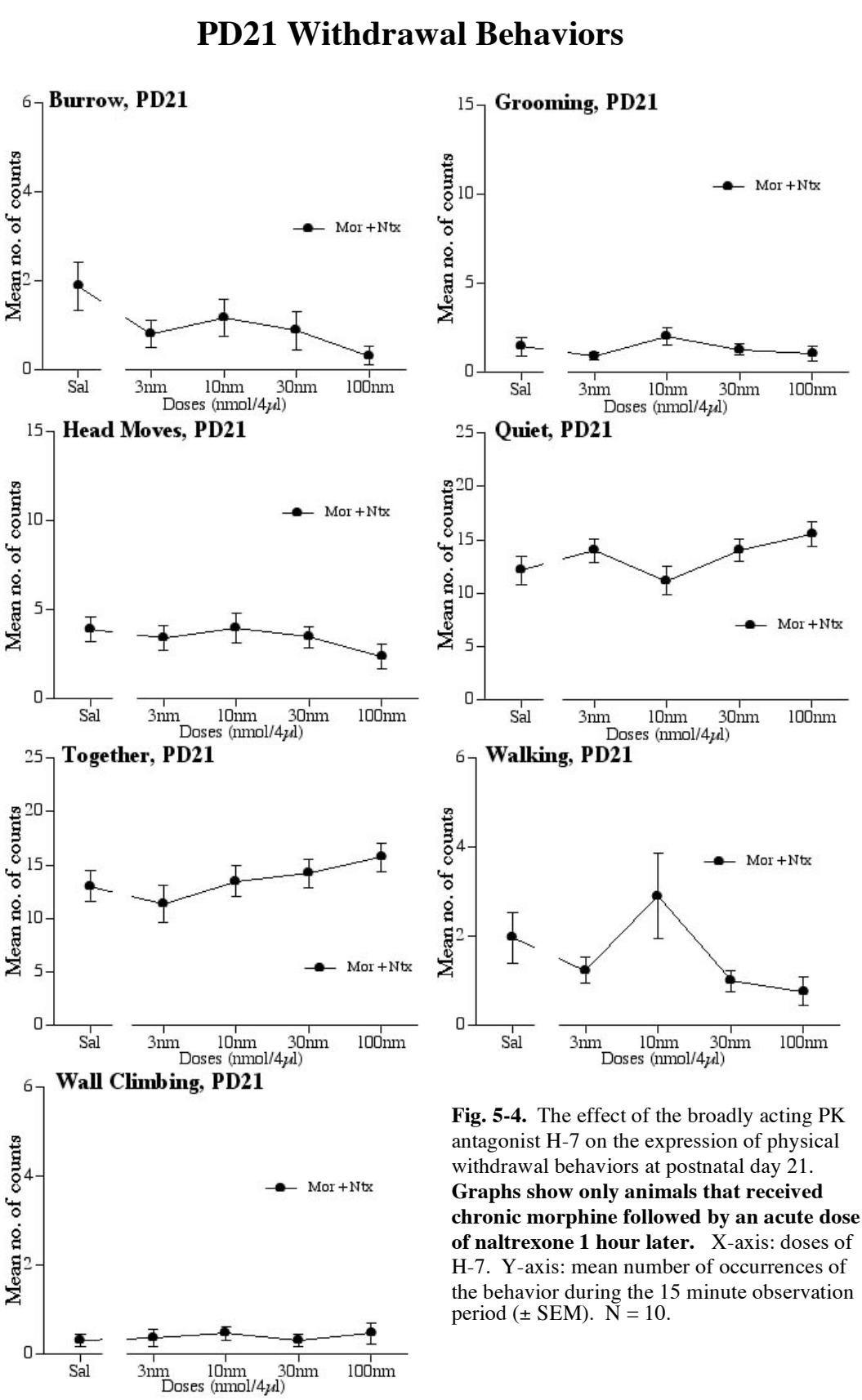


Fig. 5-4. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 21. **Graphs show only animals that received chronic morphine followed by an acute dose of naltrexone 1 hour later.** X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

Withdrawal Behavior Statistics

7do

Behavior	F	df	P
Head Moves	0.95	(4,36)	0.45
Moving Paw	0.65	(4,36)	0.63
Quiet	0.73	(4,36)	0.57
Straub	1.56	(4,36)	0.21
Twisting	1.06	(4,36)	0.39
Walking	0.75	(4,36)	0.57
Wall Climbing	1.22	(4,36)	0.32

21do

Behavior	F	df	P
Burrow	0.89	(4,36)	0.48
Grooming	0.52	(4,36)	0.72
Head Moves	0.45	(4,36)	0.77
Quiet	0.98	(4,36)	0.43
Together	0.82	(4,36)	0.52
Walking	0.96	(4,36)	0.44
Wall Climbing	0.11	(4,36)	0.98

Table 5-2. Statistical analysis of the effects of H-7 on the expression of physical withdrawal behaviors a both age groups. **Analysis was done only on the MN treatment group.** No significance was found.

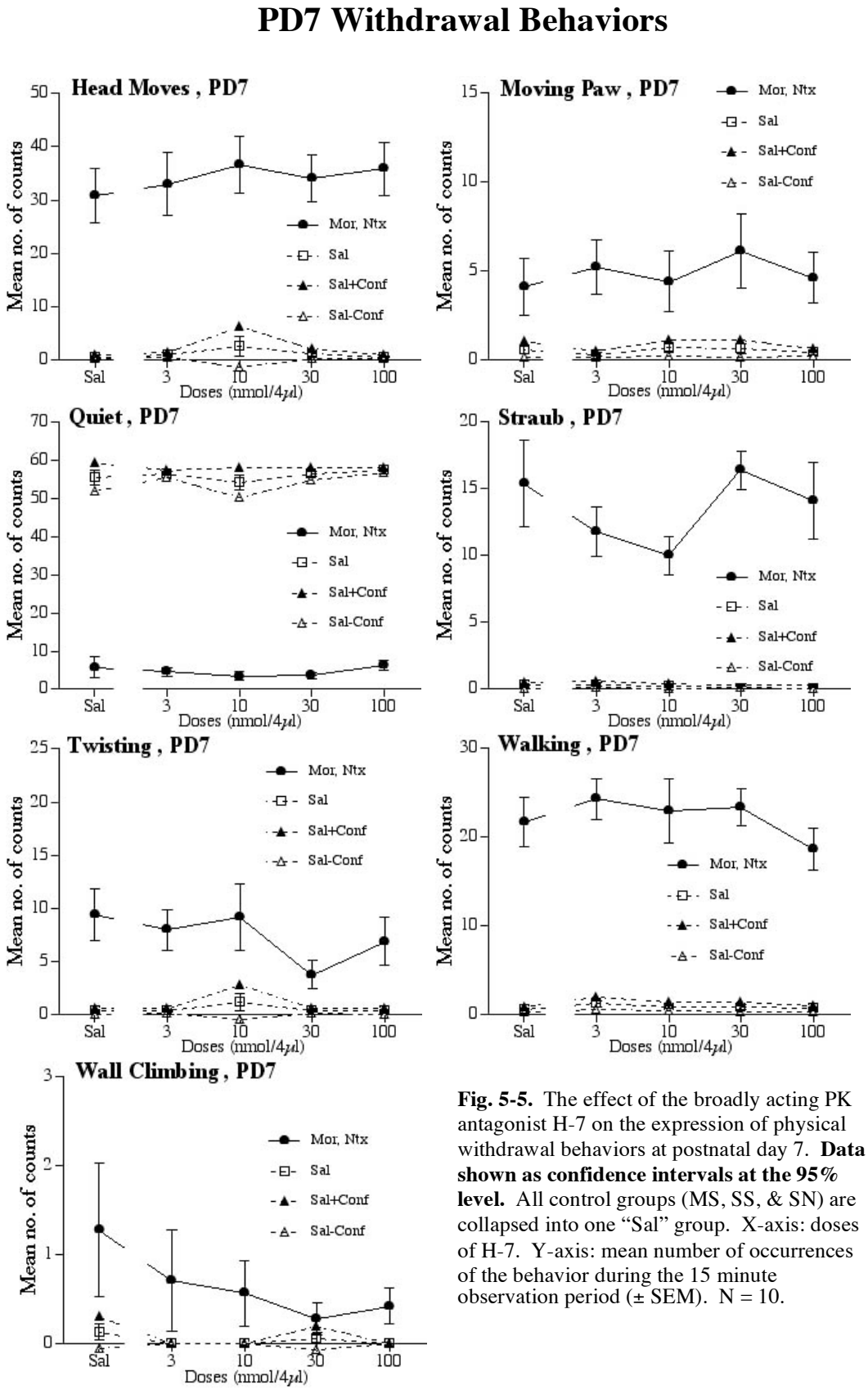


Fig. 5-5. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 7. **Data shown as confidence intervals at the 95% level.** All control groups (MS, SS, & SN) are collapsed into one "Sal" group. X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

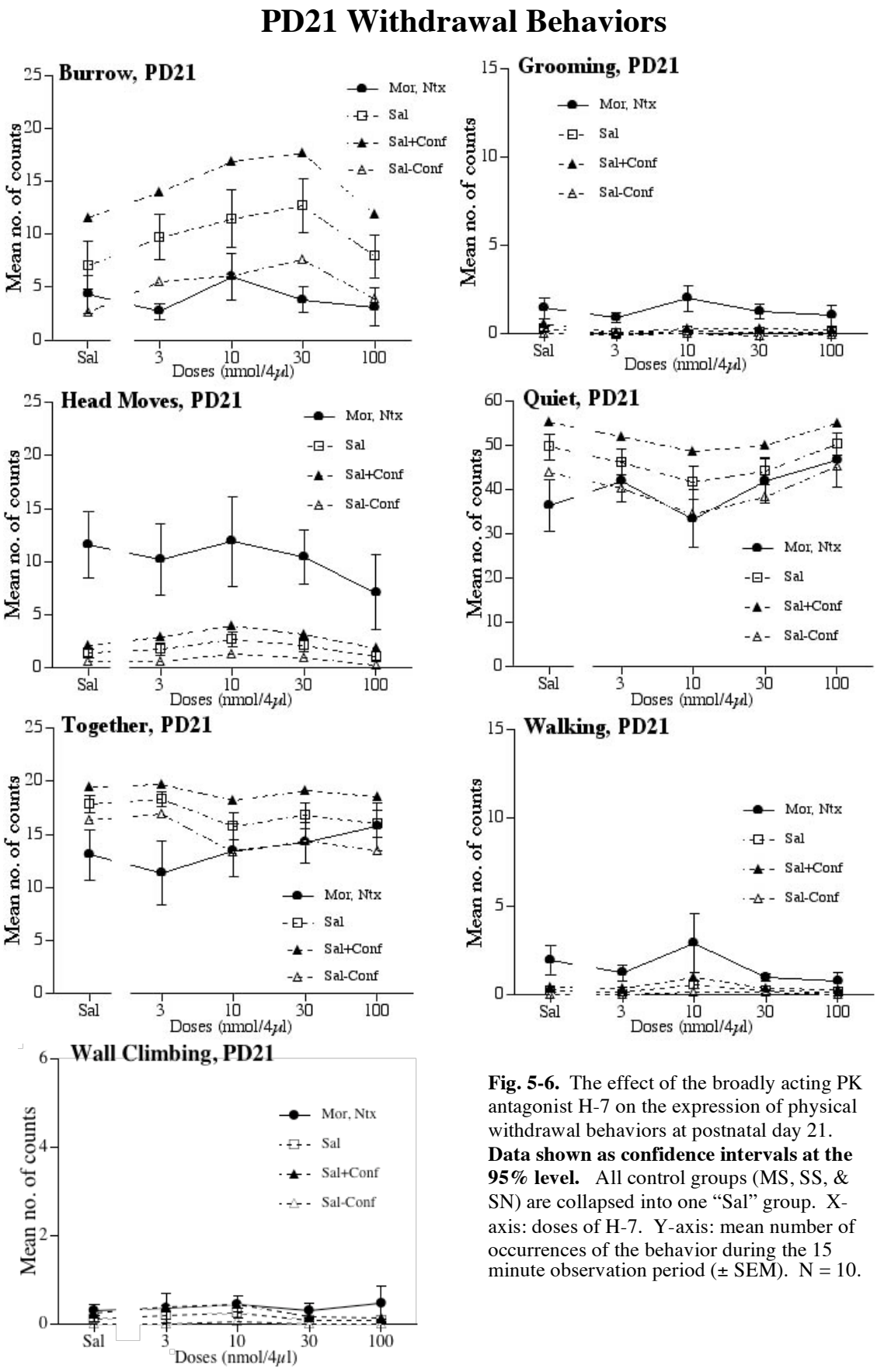


Fig. 5-6. The effect of the broadly acting PK antagonist H-7 on the expression of physical withdrawal behaviors at postnatal day 21. **Data shown as confidence intervals at the 95% level.** All control groups (MS, SS, & SN) are collapsed into one "Sal" group. X-axis: doses of H-7. Y-axis: mean number of occurrences of the behavior during the 15 minute observation period (± SEM). N = 10.

Withdrawal Behavior Mean Table

7do	H-7 Dose (nmol)				
Behavior	Sal	3	10	30	100
Sal					
Head Moves	0.6 ± .18	0.9 ± .23	2.57 ±1.95	1.13 ± .49	0.59 ± .15
Moving Paw	0.57 ± .22	0.3 ±.10	0.67 ± .23	0.62 ± .26	0.41 ± .12
Quiet	55.67 ± 1.97	56.63 ± .51	54.23 ±2.01	56.5 ± .76	57.52 ± .34
Straub	0.27 ± .13	0.33 ± .12	0.23 ± .09	0.33 ± .11	0.28 ± .12
Twisting	0.33 ± .12	0.43 ± .12	1.17 ± .83	0.4 ± .13	0.35 ± .15
Walking	0.5 ± .13	1.17 ± .36	0.83 ± .25	0.8 ± .26	0.59 ± .18
WC	0.13 ± .09	0.0	0.0	0.07 ± .07	0.0
Mor+Ntx					
Head Moves	30.8 ± 5.03	33 ± 5.98	36.6 ± 5.27	34.1 ± 4.34	35.8 ± 5.04
Moving Paw	4.1 ± 1.61	5.2 ± 1.52	4.4 ± 1.68	6.1 ± 2.09	4.6 ± 1.44
Quiet	5.7 ± 2.74	4.5 ± 1.34	3.5 ± 1.13	3.6 ± .73	6.3 ± 1.33
Straub	15.4 ± 3.27	11.8 ± 1.87	10 ± 1.45	16.4 ± 1.42	14.1 ± 2.87
Twisting	9.4 ± 2.4	8.0 ± 1.9	9.2 ± 3.12	3.8 ± 1.34	6.9 ± 2.23
Walking	21.6 ± .22	24.2 ± 2.32	22.9 ± 3.6	23.3 ± 2.08	18.6 ± 2.38
WC	1.4 ±. 6	1.0 ± .47	0.6 ± .31	0.6 ± .4	0.3 ± .15

21do	H-7 Dose (nmol)				
Behavior	Sal	3	10	30	100
Sal					
Burrow	7.07 ± 2.25	9.7 ± 2.15	11.5 ± 2.74	13 ± 2.6	7.9 ± 2.02
Grooming	0.27 ± .14	0.03 ± .03	0.14 ± .07	0.1 ± .10	0.1 ± .07
Head Moves	1.37 ± .38	1.73 ± .57	2.64 ± .68	2.07 ± .55	1.07 ± .42
Quiet	49.7 ± 2.89	46.23 ± 2.95	41.57 ± 3.66	44.2 ± 2.98	50.23 ± 2.49
Together	17.88 ± .81	18.3 ± .72	15.75 ± 1.26	16.78 ± 1.2	15.99 ± 1.31
Walking	0.22 ± .09	0.17 ± .08	0.56 ± .21	0.24 ± .06	0.14 ± .07
WC	0.13 ± .07	0.2 ± .09	0.25 ± .10	0.09 ± .04	0.07 ± .04
Mor+Ntx					
Burrow	4.3 ± 1.78	2.7 ± .76	6 ± 2.21	3.8 ± 1.21	3.1 ± 1.8
Grooming	1.44 ± .6	0.91 ± .26	2 ± .74	1.26 ± .40	1.03 ± .6
Head Moves	11.6 ± 3.16	10.2 ± 3.31	11.9 ± 4.24	10.4 ± 2.52	7.1 ± 3.53
Quiet	36.4 ± 5.78	41.9 ± 4.66	33.5 ± 6.65	42 ± 5.1	46.6 ± 6.13
Together	13.05 ± 2.35	11.34 ± 3.05	13.47 ± 2.48	14.2 ± 1.89	15.7 ± 2.25
Walking	1.96 ± .81	1.23 ± .45	2.9 ± 1.67	0.99 ± .22	0.77 ± .48
WC	0.3 ± .14	0.36 ± .33	0.46 ± .18	0.3 ± .17	0.47 ± .4

Table 5-3. Mean (± SEM) table of the effects of H-7 on the expression of physical withdrawal behaviors at both ages. **Analysis was done for confidence interval at the 95% level.** "WC" = wall climbing.

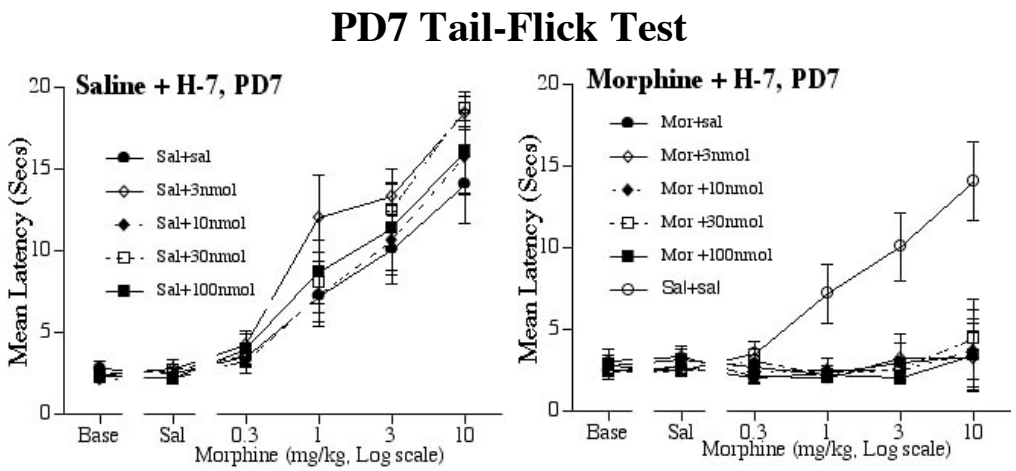


Fig 5-7A. The effect of the broadly acting PK inhibitor H-7 on the expression of tolerance at postnatal day 7. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of H-7 on PD7, and tail-flick latencies were measured 1 hour later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

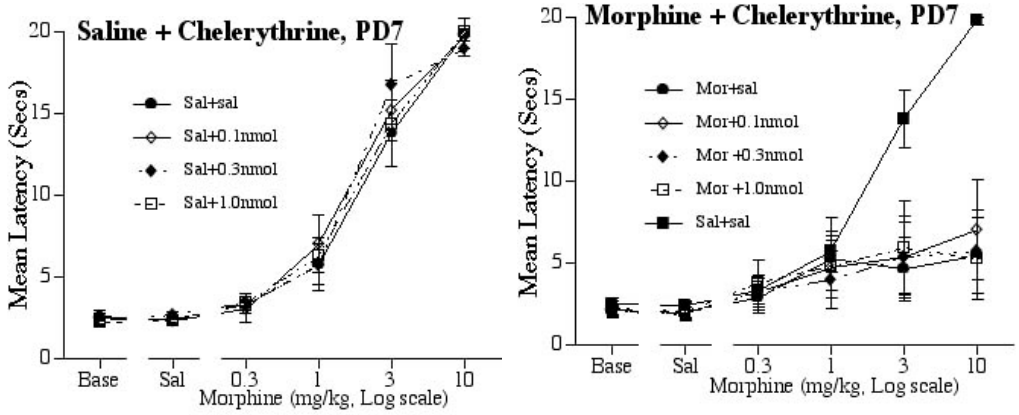


Fig 5-7B. The effect of the specific PKC inhibitor chelerythrine on the expression of tolerance at postnatal day 7. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of chelerythrine on PD7, and tail-flick latencies were measured 10 minutes later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

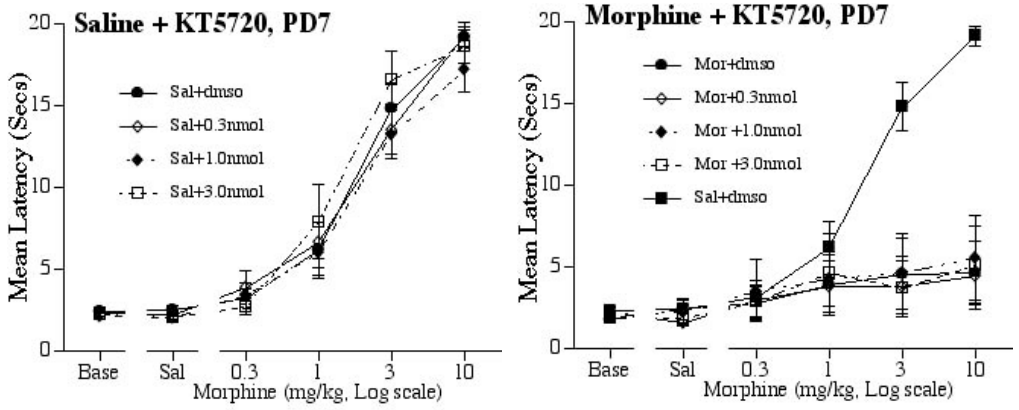


Fig 5-7C. The effect of the specific PKA inhibitor KT5720 on the expression of tolerance at postnatal day 7. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of KT5720 on PD7, and tail-flick latencies were measured 10 minutes later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

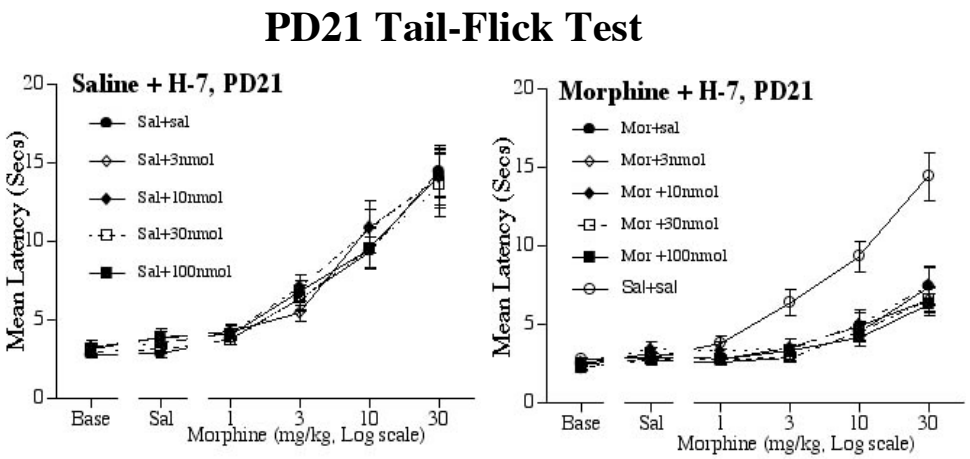


Fig 5-8A. The effect of the broadly acting PK inhibitor H-7 on the expression of tolerance at postnatal day 21. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of H-7 on PD21, and tail-flick latencies were measured 1 hour later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

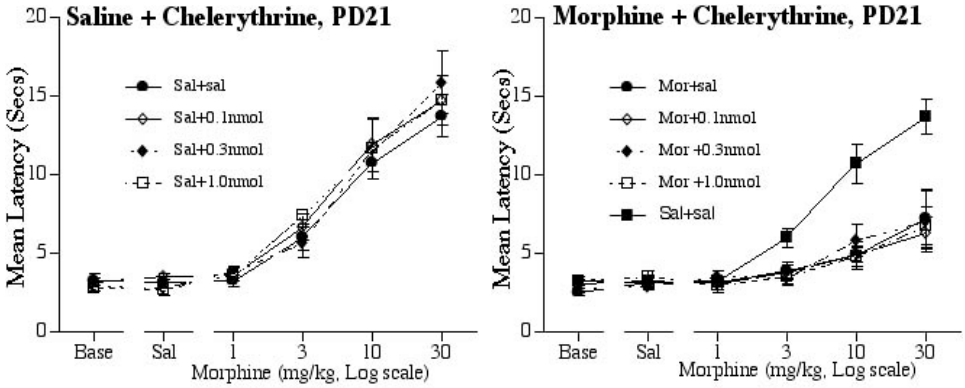


Fig 5-8B. The effect of the specific PKC inhibitor chelerythrine on the expression of tolerance at postnatal day 21. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of chelerythrine on PD21, and tail-flick latencies were measured 10 minutes later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

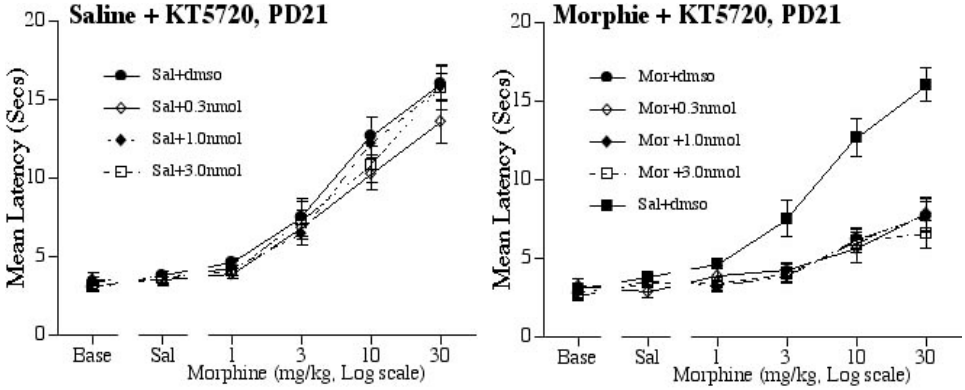


Fig 5-8C. The effect of the specific PKA inhibitor KT5720 on the expression of tolerance at postnatal day 21. Animals were treated (ip) with saline (left graph) or morphine (right graph) for 6.5 days, given an acute icv injection of KT5720 on PD21, and tail-flick latencies were measured 10 minutes later. X-axis: doses of morphine challenge. Y-axis: mean latency to withdraw tail from a heated water bath (± SEM). N = 8. Note: The Sal + sal control has been added to the chronic morphine graph for ease of comparison.

PD7 Tail-Flick Test Means

Treatment (7do)	H-7 Doses (nmol)				
	Sal	3.0	10	30	100
Sal+Base	2.74±.48	2.34 ± .26	2.11 ± .22	2.3 ± .21	2.28 ±.06
Sal+Sal	2.44 ± .3	2.75 ±.5	2.17 ±.32	2.67 ±.37	2.19 ± .19
Sal + Mor (0.3mg/kg)	3.52 ± .69	4.25 ±.77	3.19 ±.76	3.59 ±.59	3.91 ±.1
Sal + Mor (1.0mg/kg)	7.16 ± 1.83	11.97 ±2.66	7.3 ±1.64	8.02 ±1.81	8.68 ±1.94
Sal + Mor (3.0mg/kg)	10.01 ± 2.09	13.26 ±.88	10.59 ±2.16	12.51 ±2.47	11.39 ±2.63
Sal + Mor (10mg/kg)	14.06 ±2.4	18.39 ±.1	15.69 ±2.24	18.64 ±1.09	15.98 ±2.58
Mor+Base	2.5 ±.36	2.99 ± .79	2.48 ± .56	2.41 ± .52	2.75 ± .65
Mor+Sal	2.64 ±.51	3.27 ±.71	2.43 ±.32	2.38 ±.32	3.09 ±.66
Mor + Mor (0.3mg/kg)	1.99 ±.25	2.07 ±.43	2.33 ±.33	3.04 ±.71	2.68 ±.47
Mor + Mor (1.0mg/kg)	2.29 ±.42	1.98 ±.25	2.5 ±.69	2.17 ±.4	2.2 ±.36
Mor + Mor (3.0mg/kg)	2.94 ±1.17	3.19 ±1.53	2.47 ±.69	2.52 ±.62	2.0 ±.29
Mor + Mor (10mg/kg)	3.33 ± 2.04	3.19 ±1.7	3.67 ±2.5	4.37 ±2.46	3.43 ±2.15

	Chelerythrine Doses (nmol)			
	Sal	0.1	0.3	1.0
Sal+Base	2.51 ± .32	2.59 ± .45	2.26 ± .18	2.23 ± .26
Sal+Sal	2.38 ±.26	2.33 ±.28	2.61 ±.36	2.24 ±.22
Sal + Mor (0.3mg/kg)	3.31 ±.77	2.98 ±.61	3.41 ±.80	3.37 ±.50
Sal + Mor (1.0mg/kg)	5.73 ±1.25	7.01 ±1.63	5.75 ±1.73	6.22 ±1.28
Sal + Mor (3.0mg/kg)	13.78 ±1.73	15.16 ±2.07	16.75 ±1.84	14.35 ±2.46
Sal + Mor (10mg/kg)	19.77 ±.24	19.65 ±.38	18.91 ±1.16	0.0
Mor+Base	2.12 ± .27	2.08 ± .38	2.02 ± .26	2.20 ± .47
Mor+Sal	1.99 ±.34	1.93 ±.40	1.81 ±.19	2.04 ±.56
Mor + Mor (1.0mg/kg)	2.90 ±.99	3.22 ±.88	3.20 ±1.08	3.84 ±1.35
Mor + Mor (3.0mg/kg)	5.30 ±2.46	4.76 ±1.90	3.95 ±1.75	4.83 ±1.54
Mor + Mor (10mg/kg)	4.65 ±1.93	5.34 ±2.51	5.34 ±2.17	5.91 ±2.89
Mor + Mor (30mg/kg)	5.47 ±2.30	7.02 ±3.07	5.71 ±2.54	5.27 ±2.49

	KT5720 Doses (nmol)			
	DMSO	0.3	1.0	3.0
Sal+Base	2.33 ± .29	2.26 ± .31	2.11 ± .22	2.15 ± .20
Sal+Sal	2.45 ±.28	2.16 ±.25	2.01 ±.16	1.96 ±.16
Sal + Mor (0.3mg/kg)	3.20 ±.57	3.86 ±1.04	3.37 ±.77	2.68 ±.48
Sal + Mor (1.0mg/kg)	6.19 ±1.64	6.63 ±1.55	6.01 ±1.58	7.87 ±2.27
Sal + Mor (3.0mg/kg)	14.83 ±1.49	13.52 ±1.52	13.24 ±1.47	16.58 ±1.68
Sal + Mor (10mg/kg)	19.15 ±.61	19.25 ±.80	17.17 ±1.34	18.57 ±1.01
Mor+Base	1.79 ± .23	2.20 ± .28	2.11 ± .52	1.99 ± .33
Mor+Sal	2.42 ±.67	1.61 ±.21	2.27 ±.74	1.89 ±.33
Mor + Mor (1.0mg/kg)	2.81 ±.99	2.94 ±1.19	3.57 ±1.86	2.90 ±.96
Mor + Mor (3.0mg/kg)	3.91 ±1.88	3.79 ±1.55	4.27 ±1.71	4.63 ±2.42
Mor + Mor (10mg/kg)	4.58 ±2.20	3.78 ±1.86	4.61 ±2.45	3.74 ±1.60
Mor + Mor (30mg/kg)	4.66 ±1.90	4.48 ±2.09	5.56 ±2.58	5.11 ±2.43

Table 5-4. Mean (± SEM) table of the effects of each PK antagonists on analgesic tolerance to a heated water bath at PD7.

PD21 Tail-Flick Test Means

Treatment (21do)	H-7 Doses (nmol)				
	Sal	3.0	10	30	100
Sal+Base	2.72 ± .16	3.23 ±.42	3.18 ±.35	2.94 ±.29	3.12 ±.31
Sal+Sal	2.88 ±.28	3.85 ±.53	3.49 ±.34	3.11 ±.3	3.87 ±.39
Sal + Mor (1.0mg/kg)	3.8 ±.46	4.21 ±.50	4.18 ±.46	3.63 ±.25	4.07 ±.35
Sal + Mor (3.0mg/kg)	6.39 ±.84	5.4 ±.51	7.13 ±.68	6.31 ±.72	6.85 ±.65
Sal + Mor (10mg/kg)	9.29 ±1.0	10.88 ±1.64	10.83 ±1.16	9.54 ±1.36	9.49 ±1.29
Sal + Mor (30mg/kg)	14.38 ±1.51	14.02 ±1.76	14.13 ±1.39	13.61 ±2.05	14.1 ±2.02
Mor+Base	2.42 ± .29	2.17 ± .19	2.59 ± .22	2.22 ± .20	2.51 ± .21
Mor+Sal	2.63 ± .28	2.8 ±.40	3.42 ±.46	2.70 ±.25	3.12 ±.38
Mor + Mor (1.0mg/kg)	2.6 ±.23	2.71 ±.24	3.29 ±.57	2.71 ±.20	2.78 ±.22
Mor + Mor (3.0mg/kg)	2.87 ±.31	3.44 ±.63	3.38 ±.28	2.87 ±.18	3.28 ±.38
Mor + Mor (10mg/kg)	4.55 ±.61	4.89 ±1.02	4.95 ±.74	4.48 ±.61	4.10 ±.52
Mor + Mor (30mg/kg)	7.38 ±1.22	6.58 ±.80	7.47 ±1.19	6.50 ±.81	6.23 ±.68
	Chelerythrine Doses (nmol)				
	Sal	0.1	0.3	1.0	
Sal+Base	3.23 ± .30	3.18 ± .48	2.87 ± .25	2.75 ± .26	
Sal+Sal	3.13 ±.31	3.51 ±.39	2.75 ±.20	2.71 ±.43	
Sal + Mor (1.0mg/kg)	3.20 ±.14	3.65 ±.36	3.82 ±.42	3.58 ±.36	
Sal + Mor (3.0mg/kg)	6.0 ±.60	6.64 ±.86	5.69 ±.83	7.44 ±1.0	
Sal + Mor (10mg/kg)	10.71 ±1.22	11.90 ±1.01	11.60 ±1.73	11.69 ±1.88	
Sal + Mor (30mg/kg)	13.73 ±1.11	14.69 ±1.34	15.87 ±1.58	14.75 ±2.0	
Mor+Base	2.49 ± .13	3.06 ± .31	2.76 ± .30	3.27 ± .24	
Mor+Sal	3.18 ±.37	3.25 ±.40	2.87 ±.20	3.48 ±.42	
Mor + Mor (1.0mg/kg)	3.16 ±.27	3.03 ±.50	3.49 ±.44	2.93 ±.22	
Mor + Mor (3.0mg/kg)	3.88 ±.56	3.83 ±.41	3.42 ±.36	3.48 ±.49	
Mor + Mor (10mg/kg)	4.87 ±.92	4.91 ±.47	5.83 ±1.04	4.80 ±.67	
Mor + Mor (30mg/kg)	7.19 ±1.84	6.30 ±.99	7.03 ±1.96	6.75 ±1.18	
	KT5720 Doses (nmol)				
	DMSO	0.3	1.0	3.0	
Sal+Base	3.07 ± .22	3.52 ± .46	3.46 ± .31	3.12 ± .26	
Sal+Sal	3.79 ±.28	3.57 ±.36	3.72 ±.41	3.65 ±.26	
Sal + Mor (1.0mg/kg)	4.65 ±.33	3.90 ±.23	4.28 ±.45	4.08 ±.40	
Sal + Mor (3.0mg/kg)	7.56 ±1.19	6.88 ±1.09	6.47 ±.67	7.38 ±1.20	
Sal + Mor (10mg/kg)	12.69 ±1.20	10.32 ±1.0	12.26 ±1.67	10.88 ±1.16	
Sal + Mor (30mg/kg)	16.06 ±1.08	13.65 ±1.39	15.88 ±.87	15.81 ±1.43	
Mor+Base	2.75 ± .23	3.23 ± .44	2.80 ± .38	2.57 ± .27	
Mor+Sal	3.56 ±.41	2.88 ±.37	3.54 ±.35	3.40 ±.21	
Mor + Mor (1.0mg/kg)	3.29 ±.33	3.94 ±.61	3.23 ±.32	3.39 ±.38	
Mor + Mor (3.0mg/kg)	3.94 ±.37	4.30 ±.41	3.94 ±.51	4.02 ±.60	
Mor + Mor (10mg/kg)	6.25 ±.62	5.62 ±.85	6.13 ±.56	6.15 ±.77	
Mor + Mor (30mg/kg)	7.74 ±1.04	7.84 ±1.05	7.68 ±.98	6.55 ±.85	

Table 5-5. Mean (± SEM) table of the effects of each PK antagonists on analgesic on tolerance to a heated water bath at P21.

Chapter 6: The Modulation of Protein Kinases by Morphine Treatment (Molecular Analysis)

Introduction

The powerful analgesic and euphoric effects of opiates have made them highly effective for the treatment of chronic pain in the clinic, as well, as popular in the illegal street market. An unfortunate side effect of these drugs is that chronic exposure to them results in tolerance and dependence. As a result of these adaptations, more of the drug is needed to exert the same effect and its discontinuation results in unpleasant (but rarely life-threatening) withdrawal symptoms.

The molecular and cellular mechanisms that modulate tolerance and dependence are still not fully understood. There is evidence that changes in opioid receptor (μ, κ, and δ) density or function and their subsequent activation of G_i and G_o proteins regulates tolerance and dependence (Trujillo and Akil 1991; Pasternak, Kolesnikov et al. 1995; Sim-Selley, Selley et al. 2000; Liu and Anand 2001; Yoburn, Gomes et al. 2003). However, this does not appear to be the entire picture because the findings are conflicting (Nestler and Tallman 1988; Rasmussen, Beitner-Johnson et al. 1990; Fleming, Ponjee et al. 1992; Belanger, Ma et al. 2002) and the changes in receptor function are too short

lived to explain the long-term alterations in protein synthesis and cellular activity that occur after prolonged opiate exposure.

Additional research has focused on the third messengers cAMP-dependent protein kinase (PKA) and protein kinase (PK) C (since the phosphorylation of proteins by protein kinases is one of the final common pathways by which extracellular signals alter neuronal functioning (Nestler and Tallman 1988; Fleming, Ponjee et al. 1992)) and their pathways cAMP/adenylyl cyclase and IP_3-DAG, respectively. Based on work done using cell lines, Sharma et al (Sharma, Klee et al. 1975) and Rasmussen et al (Rasmussen, Beitner-Johnson et al. 1990) theorized that initial opiate exposure reduces cellular activity through the activation of opioid receptors that are coupled to G_i or G_o proteins, which then reduces levels of the downstream proteins adenylyl cyclase and cAMP. After a period of chronic exposure, compensatory mechanisms take over, and cellular activity returns back up to control levels. If the opiate is then suddenly removed by administration of an opiate antagonist (or spontaneously) cellular activity dramatically increases above control, and levels of adenylyl cyclase and cAMP are subsequently increased.

More current work using animal models has found that within the locus coeruleus acute exposure to morphine causes a decrease in the levels of PKA (Duman, Tallman et al. 1988), whereas chronic exposure to opiates results in increases in the levels of PKA (Nestler and Tallman 1988; Nestler, Alreja et al. 1994) in the same brain region. In the pons/medulla region of rats chronically receiving opiates, there is an increase in the activity of PKC located within the cytosol (Narita, Makimura et al. 1994c; Tokuyama, Feng et al. 1995a), as well as an increase in the translocation of PKC from the cytosol to

the membrane within the spinal cord (Mayer, Mao et al. 1995). In addition, repeated intrathecal administration of morphine increases PKCγ and PKCα immunoreactivity (an effect that is reversed by an acute dose of a PKC antagonist) (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000), and increases PKC phosphorylation within the spinal cord (Granados-Soto, Kalcheva et al. 2000).

However, another study found a significant decrease in the immunoreactivity of the PKCαβ isoforms in the frontal cortex (FC) of both humans who chronically used heroin and rats who were chronically exposed to morphine (Busquets, Escriba et al. 1995; Garcia-Sevilla, Ventayol et al. 1997). The discrepancies between the changes in levels of PKC may be the result of the different anatomical locations that were studied and/or the different isoforms measured. For instance, PKCγ is found in high concentrations in the spinal cord (Mao, Price et al. 1995; Granados-Soto, Kalcheva et al. 2000), whereas in the mammalian brain PKCα is the more abundant (Busquets, Escriba et al. 1995; Garcia-Sevilla, Ventayol et al. 1997) of the PKC isoforms.

The body of research that has focused on the role that morphine exposure plays on protein kinase levels in the developing rat has made progress, but still requires more studies. Steingart et al (1998) found that mice prenatally exposed to heroin showed increased levels of total membrane PKC activity in the hippocampus when measured at postnatal day (PD) 50, an effect that can be reversed by grafting new cells into the area (Steingart, Silverman et al. 2000b). In addition, basal activity of the PKC isoforms PKCα and PKCγ remain unchanged by prenatal heroin exposure, while the cholinergic receptor induced translocation and activation of PKCγ and PKCβII is lost (Shahak, Slotkin et al. 2003; Yaniv, Naor et al. 2004). At this time, however, there is still little

information on the effects of opiate exposure on the rodent during early development (before PD21).

The following set of experiments was specifically designed to add to this body of literature by determining the role that acute and chronic morphine exposure, as well as, precipitated morphine withdrawal have on levels of PKA and PKC in the young rat. All of which are different treatment paradigms that are seen in the clinical setting and in the adult rodent literature. Rat pups were given an acute injection of morphine, chronically treated with morphine alone, or chronically treated with morphine and given an acute injection of an opiate antagonist. On PD7 or 21 their brains and spinal cords were removed and protein levels of PKA (regulatory subunits i and ii) and PKC were determined using quantitative western analysis. It was hypothesized that at PD21 acute treatment would decrease levels of PKA and PKC, chronic treatment would bring the levels back up to control levels, and withdrawal would increase the levels further; all results that were hypothesized by Rasmussen and Sharma (Sharma, Klee et al. 1975; Rasmussen, Beitner-Johnson et al. 1990) in the adult. It was also hypothesized that the animals at PD7 would have a different pattern as a result of a less developed CNS but the direction in which the effects on PKA and PKC would go was hard to predict.

Materials and Methods

Subjects/Tattooing

As described in the general methods section.

Drug and Antibody Preparation.

Drugs. All drugs were reconstituted using dH_2O. Morphine sulfate (Henry Schein) was the opiate antagonist and naltrexone hydrochloride (Sigma-Aldrich) was the broadly

acting opiate antagonist used to precipitate withdrawal. *Antibodies.* All antibodies were diluted with TBS-T (Tris buffered saline with 0.1% Tween) + 5% nonfat dried milk. The PKC (Santa Cruz, H-300) antibody was diluted 1:500 and its rabbit (Amersham Biosciences) secondary antibody was diluted 1:1000. The PKAri (BD Transduction Laboratories, 610166) and PKArii (Upstate, 06-411) antibodies were both diluted 1:500. Their associated secondary antibodies, mouse (Amersham Biosciences) and goat (Santa Cruz), respectively, were diluted 1:1000.

Treatment Schedules.

Acute and chronic morphine treatment, as well as withdrawal precipitation were performed as described in the general methods section. For all treatment schedules, two hours after the final morphine/saline injection pups were sedated with metofane and decapitated. Their brains and spinal cords were then immediately removed and quick frozen in dry ice. In the case of animals going through withdrawal, one millimeter sections of the PAG and NAcc were later punched from the frozen brain samples.

Immunoblotting.

Whole brains (acute and chronic treatment) or punched areas (withdrawal) and the spinal cords were homogenized in 20mM Hepes (7.9), 10mM KCl, 1mM EDTA, 0.2% NP40, 10%Glycerol, 100mMNaCl, and the protease inhibitors Pepstatin A (1μl/1ml), Leupeptin (1μl/1ml), DTT (1μl/1ml), Aprotinin (1μl/1ml), and PMSF (20μl/1ml). The samples were centrifuged for 15 min at 4°C. Fifty micrograms of the resulting supernatant was combined with a dye (1μl 2-mercaptoethanol: 19μl laemmli sample buffer) and boiled for 3 min. The amount of protein sample was calculated using a Bradford assay. The resulting solution was loaded in precast 4-15% Acrylamide gels,

1mm thick (Bio-Rad; 161-1104) and submitted to electrophoresis (SDS-polyacrylamide gel electrophoresis). Electrophoresis of gels was at a constant voltage (100V) for 1.5 - 2hrs and the transfer of the protein samples to a nitrocellulose membrane (Bio-Rad; 162-0145) was for 3 hours, at a constant current of .44 mA. The nitrocellulose membranes were blocked overnight at 4°C in TBS-T + 5% nonfat dried milk.

The next day the membranes were washed twice in TBS-T, followed by a 1 hour incubation in the primary antibody with TBS-T + 5% nonfat dried milk at room temperature. After the primary incubation, membranes were washed multiple times in TBS-T and incubated in the secondary antibody for another hour (room temperature). Membranes were washed further and immunoreactivity was detected with enhanced chemiluminescence (ECL) western blot detection reagents for 1 minute (Amersham; RPN2108). Molecular weights were determined using Magic Mark (Invitrogen; LC5602) and Kaleidescope Prestained Standard (Bio-Rad; 161-0324).

Quantification

Blots of the protein samples were visualized by exposure onto X-Omatic AR-5 film (Fisher Scientific) and then scanned into a computer using the Personal Densitometer SI (Molecular Dynamics). The optical densities (OD) for the blots were determined using the Image Quant 5.0 program. Values for each blot were normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH; protein OD/GAPDH OD).

Results

A one-way analysis of variance (ANOVA) was used to determine the effects of acute and chronic treatment of morphine, as well as withdrawal, on the levels of PKA and PKC. For acute treatment, morphine was the within measurement. For chronic treatment

morphine was the between measurement. For withdrawal morphine was the between measurement and naltrexone was the within measurement.

Protein Kinase Levels after Acute Morphine Treatment.

PD7: Acute morphine treatment had no significant effect on the protein levels of PKAri, PKArii, or PKC in the brain and spinal cord of rats at postnatal day 7. See figure 6-1; table 6-1. *PD21:* Acute morphine treatment had no effect on the protein levels of PKAri, PKArii, or PKC in the brain and spinal cord of rats at postnatal day 21. See figure 6-2; table 6-1.

Protein Kinase Levels after Chronic Morphine Treatment.

PD7: Chronic morphine treatment had no significant effect on the protein levels of PKAri, PKArii, or PKC in the brain and spinal cord of rats at postnatal day 7. See figure 6-3; table 6-2. *PD21:* Chronic morphine treatment had no effect on the protein levels of PKAri, PKArii, or PKC in the brain and spinal cord of rats at postnatal day 21. See figure 6-4; table 6-2.

Protein Kinase levels after Morphine Withdrawal.

PD7: Precipitated morphine withdrawal had a significant effect on protein levels of PKArii and PKC in the PAG, but no effect on PKAri, PKArii, or PKC protein levels in the SC or NAcc, or PKAri protein levels in the PAG at postnatal day 7. See figure 6-5; table 6-3. *PD21:* Precipitated morphine withdrawal had a significant effect on protein levels of PKArii in the NAcc. It had no effect, however, on PKAri, PKArii, or PKC protein levels in the SC or PAG, or PKAri and PKC protein levels in the NAcc at postnatal day 21. See figure 6-6; table 6-3.

Discussion

The goal of the experiments described in this chapter was to determine if exposure to morphine would alter the protein levels of PKA and PKC in the young rodent. Although an antibody for whole PKA could not be obtained at the time the experiments were performed, effects on the two regulatory subunits of PKA and whole PKC were chosen as an adequate starting pointing for this area of research. To determine this, rats were given a single dose of morphine, chronic injections of morphine alone, or chronic injections of morphine followed by an acute injection of naltrexone. On PD7 or 21 rat pups were sacrificed, their brains and spinal cords were removed, and the tissue was processed for quantitative western analysis. For both ages, acute and chronic treatment of morphine did not alter protein levels of PKAri, PKArii, or PKC, as compared to control levels. See figures 6-1 thru 6-4; tables 6-1 and 6-2. Precipitated withdrawal altered protein levels of PKArii and PKC in the PAG at PD7 and protein levels of PKArii in the NAcc at PD21, as compared to controls. All other protein levels remained unaffected at both age groups during withdrawal. See figures 6-5 and 6-6; table 6-3.

Protein Kinase levels after acute Morphine Treatment.

Opioid receptors are coupled to both the IP_3-DAG and cAMP/adenylyl cyclase system via a G_i (or G_o) protein. As a result, it has been hypothesized that the acute activation of opioid receptors by an agonist results in decreased activity and/or levels of all the downstream components (including effectors, second messengers, protein kinases, and genes). In addition, *in vivo* and *in vitro* studies have found evidence for this (Sharma, Klee et al. 1975; Nestler and Tallman 1988; Rasmussen, Beitner-Johnson et al. 1990;

Ventayol, Busquets et al. 1997). Therefore, the fact that PKA and PKC remained unaffected at PD21 in the current experiment was unexpected.

The paradigm was adapted from the literature for use with younger rodents and it may have required further modifications. For instance, the dose of morphine given is a standard dose in our laboratory for chronic treatment, but it may not have been high enough to cause an effect after only one treatment. In addition, the two-hour delay before the animals were sacrificed may have been too long; although it is a common time length in the adult literature (Duman, Tallman et al. 1988; Nestler and Tallman 1988; Nestler, Erdos et al. 1989; Busquets, Escriba et al. 1995; Ventayol, Busquets et al. 1997) not all of the studies focused solely on protein kinases and they may have adjusted their time schedules for the other molecules of interest.

There is also the possibility that the results are exactly as they appear, at PD21 the acute treatment of morphine has no detectable effect on protein levels of PKA and PKC as measured by quantitative western analysis. Each study in the literature used a different paradigm and therefore results could only be interpreted as they related to the paradigm of interest. In the brainstem (Bernstein and Welch 1997) and the LC (Nestler and Tallman 1988) the activity of PKA remains unaffected. This is also the case for PKCαβ in the FC (Busquets, Escriba et al. 1995) and PKCγ in the dorsal horn of the SC (Mao, Price et al. 1995). However, using translocation of PKC from the cytosol to the membrane as an indicator, acute treatment with an opiate agonist increases activity of whole PKC (Kramer and Simon 1999b) and PKCβII (Harlan, Kailas et al. 2004), results that are inconsistent possibly because some authors investigated changes in protein levels while others focused on changes in activity levels.

The results for PD7 were not unexpected because research in our laboratory has shown that data from this age group mirrors the adult literature at times, but at other times does not. Chapter 3 of this dissertation describes experiments in which the predominant opioid receptor (μ-OR) active during morphine withdrawal at PD7 is the same as that seen in the adult rodent (McPhie and Barr 2000) and chapter 4 shows that a selection of brain regions that become active during opiate withdrawal at PD7, are the same that show activity in the adult rat (McPhie and Barr 2003). However, another study done in our laboratory shows that withdrawal is precipitated by microinjection of an opiate antagonist into the LC and PAG, but not into the amygdala (Jones and Barr 2001), a brain region that precipitates withdrawal in the adult. In addition, NMDA receptor antagonists, which attenuate opiate withdrawal in adult rodents, have been shown to be ineffective at PD7, partially effective at PD14 and not fully effective until PD21 (Zhu and Barr 2001a; Zhu and Barr 2003a).

Protein Kinase levels after Chronic Morphine Treatment.

To date, the greatest amount of research has focused on the effects of chronic opiate treatment on the levels and activity of PKA and PKC. The fact that the current experiments resulted in unaffected levels of the two protein kinases at PD7 and 21 after chronic morphine exposure was expected in one respect because these results can be interpreted as a return to control levels (See figure 2-3). In the cell, long-term exposure to an opiate renders it tolerant to the inhibitory effects of acute treatment and causes the levels of proteins or amount of activity to return back (up) to control levels.

However, it is still important to note the conflicting findings of the current literature on the modulation of protein kinases. Chronic opiate exposure reduces the phosphorylation

of μ-opioid receptors by PKA (Bernstein and Welch 1998), a finding that is opposite to the views of this dissertation. Other studies have found that PKA activity is increased in the LC (Nestler and Tallman 1988) and SC, NAcc, amygdala, and thalamus (Terwilliger, Beitner-Johnson et al. 1991), but not the FC, caudate/putamen, dorsal raphe nucleus, hippocampus, cerebellum, substantia nigra, PAG, and VTA.

In vivo studies with PKC show that chronic DAMGO (a μ-OR agonist) exposure decreases PKC activity (Kramer and Simon 1999b), while chronic morphiceptin increases PKCε activity (Mangoura and Dawson 1993). The *in vitro* PKC studies are not consistent, just as the PKA studies. For instance, whole PKC activity in the cytosol of the SC (Mayer, Mao et al. 1995; Li and Roerig 1999) and brain (Narita, Feng et al. 1994b; Narita, Makimura et al. 1994c; Tokuyama, Feng et al. 1995a) are increased, as well as levels of the PKCγ subunit in the SC (Mao, Price et al. 1995; Narita, Mizoguchi et al. 2001a; Narita, Suzuki et al. 2004b). However, other studies have found a decrease in the levels of PKCαβ in the FC of humans (Busquets, Escriba et al. 1995), the FC, brainstem, and hypothalamus of rats (Ventayol, Busquets et al. 1997), as well as cytosolic PKCα, PKCβI, and PKCγ in the SC (Li and Roerig 1999).

With PKC the effects of chronic treatment appear to be highly dependent on the brain region studied and the isoforms that are being probed, whereas with PKA the effect is more dependent on the brain region. In addition agonist stimulation of μ-opioid receptors by PKA can not be seen without the addition of the catalytic subunits (Chakrabarti, Law et al. 1998a), suggesting there may also be different effects when comparing the regulatory and catalytic subunits.

Protein Kinase levels after Morphine Withdrawal.

Withdrawal is a complete disruption of the homeostatic system that the neuron reestablishes after it is initially disrupted by the acute administration of a exogenous drug (Hardman, Limbird et al. 2001; Nestler 2001; Dalton, Smith et al. 2005; McClung, Nestler et al. 2005). As such, one would expect its occurrence to cause a dramatic up- or down-regulation of the IP_3-DAG and cAMP/adenylyl cyclase pathways. In the case of opiates, precipitated withdrawal (and to a lesser extent spontaneous withdrawal) has been shown to dramatically up-regulate the cAMP/adenylyl cyclase pathways (Sharma, Klee et al. 1975; Sharma, Klee et al. 1977; Nestler and Tallman 1988; Rasmussen, Beitner-Johnson et al. 1990; Benavides, Laorden et al. 2003).

It was interesting, however, that at PD7 only PKArii and PKC in the PAG and at PD21 only PKArii in the NAcc show a significant effect of precipitated opiate withdrawal in the current experiments. Rasmussen (Rasmussen, Beitner-Johnson et al. 1990) found an up-regulation of PKA levels in the LC that lasted up to 6 hours after precipitation. Another study found that PKA activity in cell cultures that is increased after acute morphine treatment, is reduced during precipitated withdrawal (Chakrabarti, Law et al. 1998a). Finally, there is a rapid increase in protein (Busquets, Escriba et al. 1995; Ventayol, Busquets et al. 1997) and mRNA (Ventayol, Busquets et al. 1997) levels of PKC$\alpha\beta$ in the FC of rats during opiate withdrawal.

When collecting the current data, it was assumed that because precipitated withdrawal is such a dramatic alteration in the cell there would be an obvious effect. This is clearly not the case since an effect is seen in only 22% of the treatment groups at PD7 and 11% of the treatment groups at PD21. This may be explained by the fact that the tissue

samples punched from each brain region were extremely small at those two age groups and were therefore not able to yield changes robust enough to detect. Perhaps multiple animals from a litter should have been pooled together to make one data point.

Another possibility is that because of the relative immaturity of the CNS of rat pups at PD7, and to some extent PD21, the effect of withdrawal on PKA and PKC levels was incomplete. For instance, by PD7 adenylyl cyclase, most PKC isoforms, opioid receptors, and G-proteins are detectable (Hashimoto, Ase et al. 1988; Rius, Barg et al. 1991a; Rius, Streaty et al. 1991b; Jiang, Naik et al. 1994; Ihnatovych, Novotny et al. 2002b), but they are not necessarily functional as evidenced by a study that showed only weak μ-OR and G-protein coupling at PD7 (Windh and Kuhn 1995a).

One final observation worth noting is the appearance of doublets for PKAri after acute morphine treatment in the brain and spinal cord at PD7 and 21 (See figures 6-1 and 6-2), after chronic morphine treatment in the spinal cord at PD21 (See figure 6-4), and after precipitated withdrawal in the spinal cord at PD7 and 21 (See figures 6-5 and 6-6). At this time it is not clear why the doublets appear primarily in the spinal cord, but there does not appear to be a differential effect in the spinal cord vs. the whole brain, NAcc and PAG for either subunit. Both regulatory subunits of PKA (i and ii) can be further subdivided into α and β isoforms (Heller, Vigil et al. 2004; Tasken and Aandahl 2004; Kim, Xuong et al. 2005) and it is very likely that the doublets represent these two isoforms for the PKAri subunit. The reason they do not appear for PKArii is unclear, but there is data showing that each subunit and isoform has specific functions (Heller, Vigil et al. 2004; Tasken and Aandahl 2004) and localizations (Hausken, Coghlan et al. 1994; Tasken and Aandahl 2004). Therefore it is possible that the current findings represent

the end result of different functions of PKAri and PKArii that only became apparent with opiate treatment. Although this cannot be verified at this time based on the experiments conducted in this dissertation, it may be something worth pursuing in future experiments.

Overall, the findings of this chapter were unexpected, but promising. It is important to establish the fact that young rodents can be tested in paradigms similar to those used for adult rodents. The next step will be to continue to modify the paradigm to accurately detect the treatment effects. Also, to the best of my knowledge, this is one of only a handful of experiments describing opiate regulation of PKA and PKC levels during early ontogeny. This is important because the IP_3-DAG and cAMP/ adenylyl cyclase signal transduction systems are important systems for normal neuronal communication. In addition, the efforts to produce drugs that are able to mimic the acute effects of opiates, without the side effects of chronic opiate exposure, are focusing on the intracellular (and molecular) mechanisms that underlie behaviors. It is very likely these new compounds will be used as drug therapies for infants and as a result it is important to understand how they interact with the developing CNS.

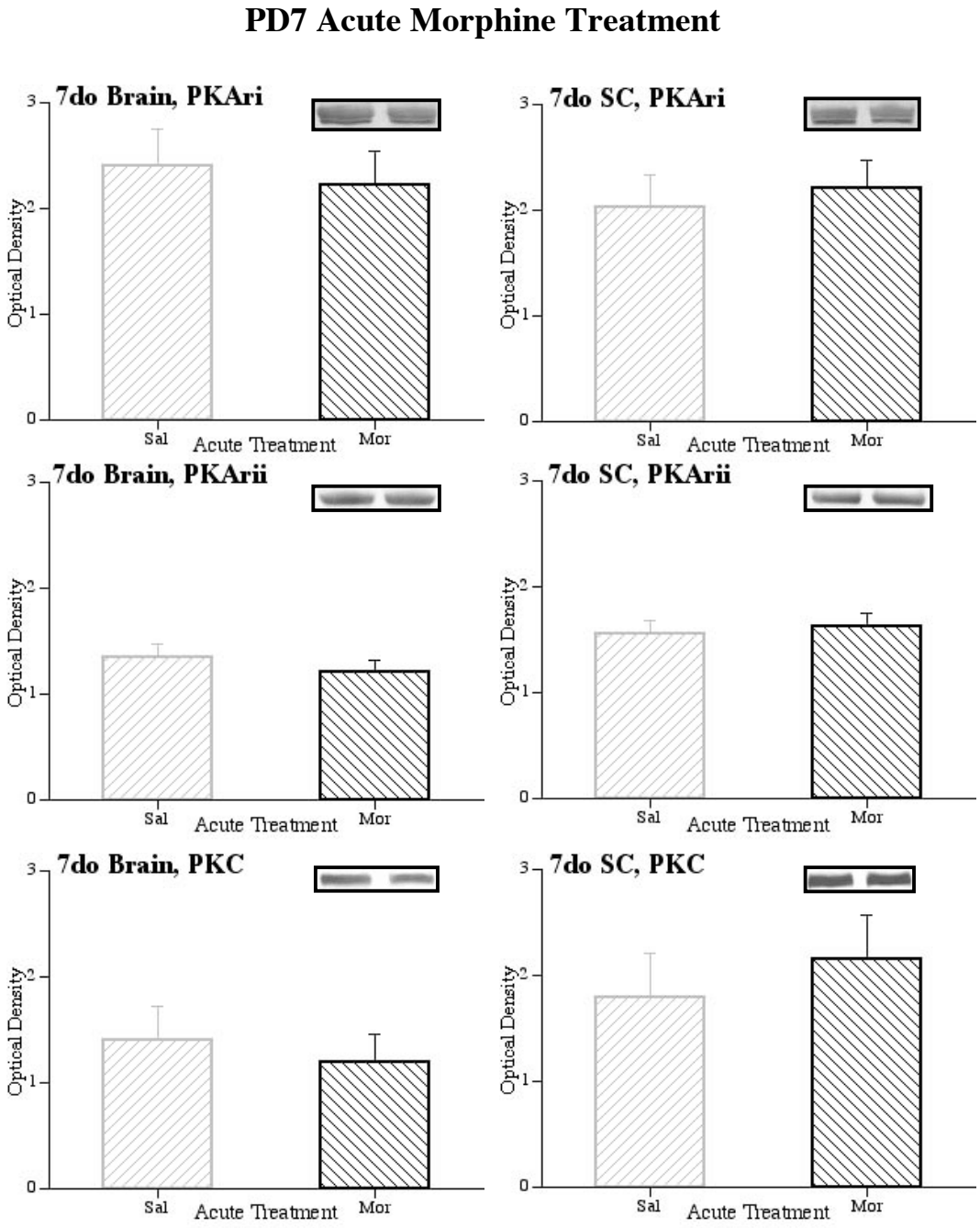


Fig 6-1. The effect of **acute** morphine treatment on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 7. Animals were treated (ip) with one injection of saline or morphine, two hours later their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: acute treatment (10mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 6. Boxes show a representative western blot.

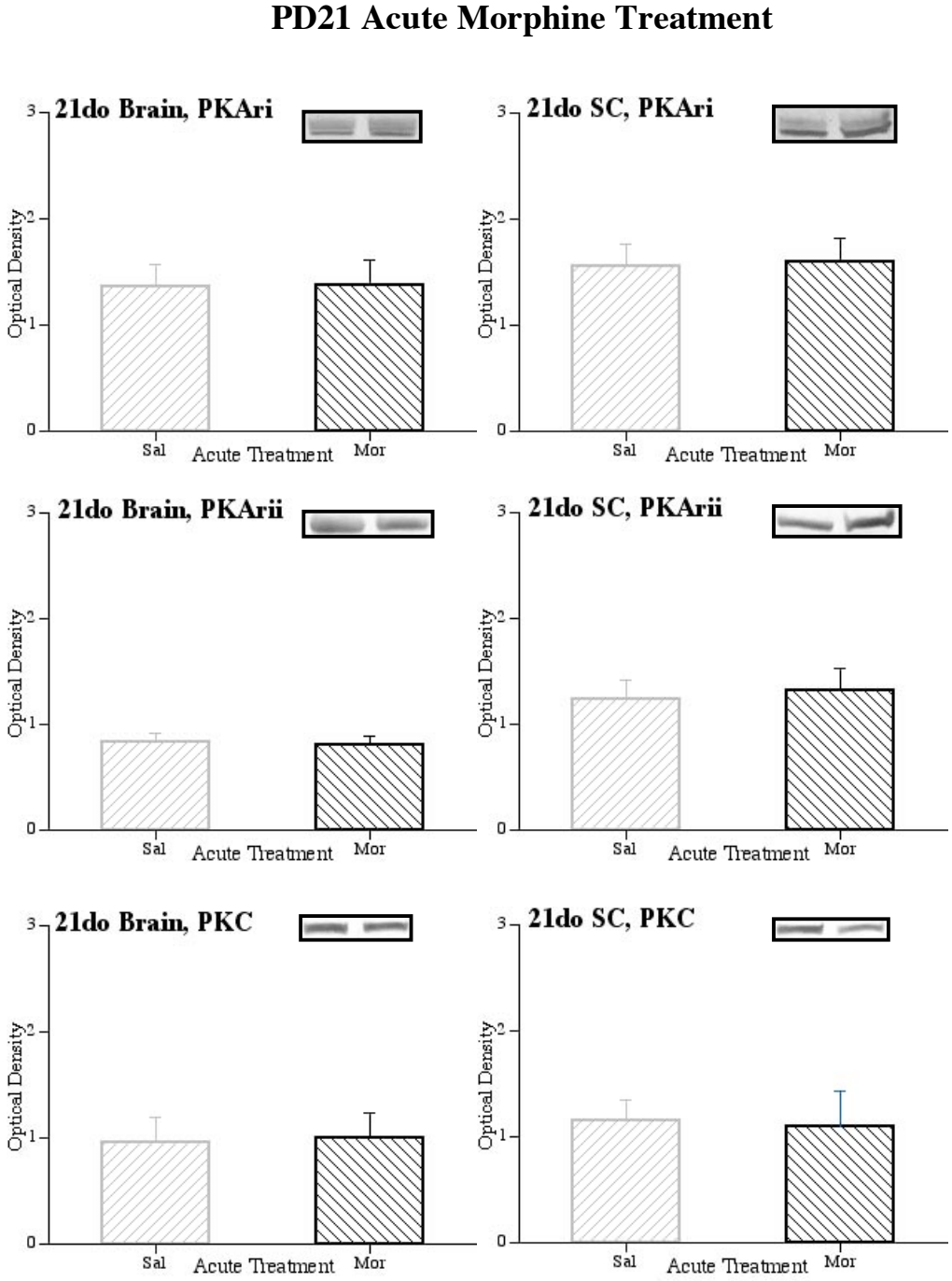


Fig 6-2. The effect of **acute** morphine treatment on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 21. Animals were treated (ip) with one injection of saline or morphine, two hours later their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: acute treatment (10mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 6. Boxes show a representative western blot.

Acute Morphine Treatment Statistics

Brain

Age/Protein	F	df	P
7do/PKAri	0.14	(1,10)	0.72
7do/PKArii	0.62	(1,10)	0.45
7do/PKC	0.23	(1,10)	0.64
21do/PKAri	0.001	(1,10)	0.97
21do/PKArii	0.08	(1,10)	0.78
21do/PKC	0.01	(1,10)	0.92

Spinal cord

Age/Protein	F	df	P
7do/PKAri	0.21	(1,10)	0.65
7do/PKArii	0.17	(1,10)	0.69
7do/PKC	0.37	(1,10)	0.56
21do/PKAri	0.02	(1,10)	0.91
21do/PKArii	0.12	(1,10)	0.74
21do/PKC	0.02	(1,10)	0.89

Table 6-1. Statistical analysis of **acute** morphine treatment on levels of each of the PKs at each age group. No significance was found.

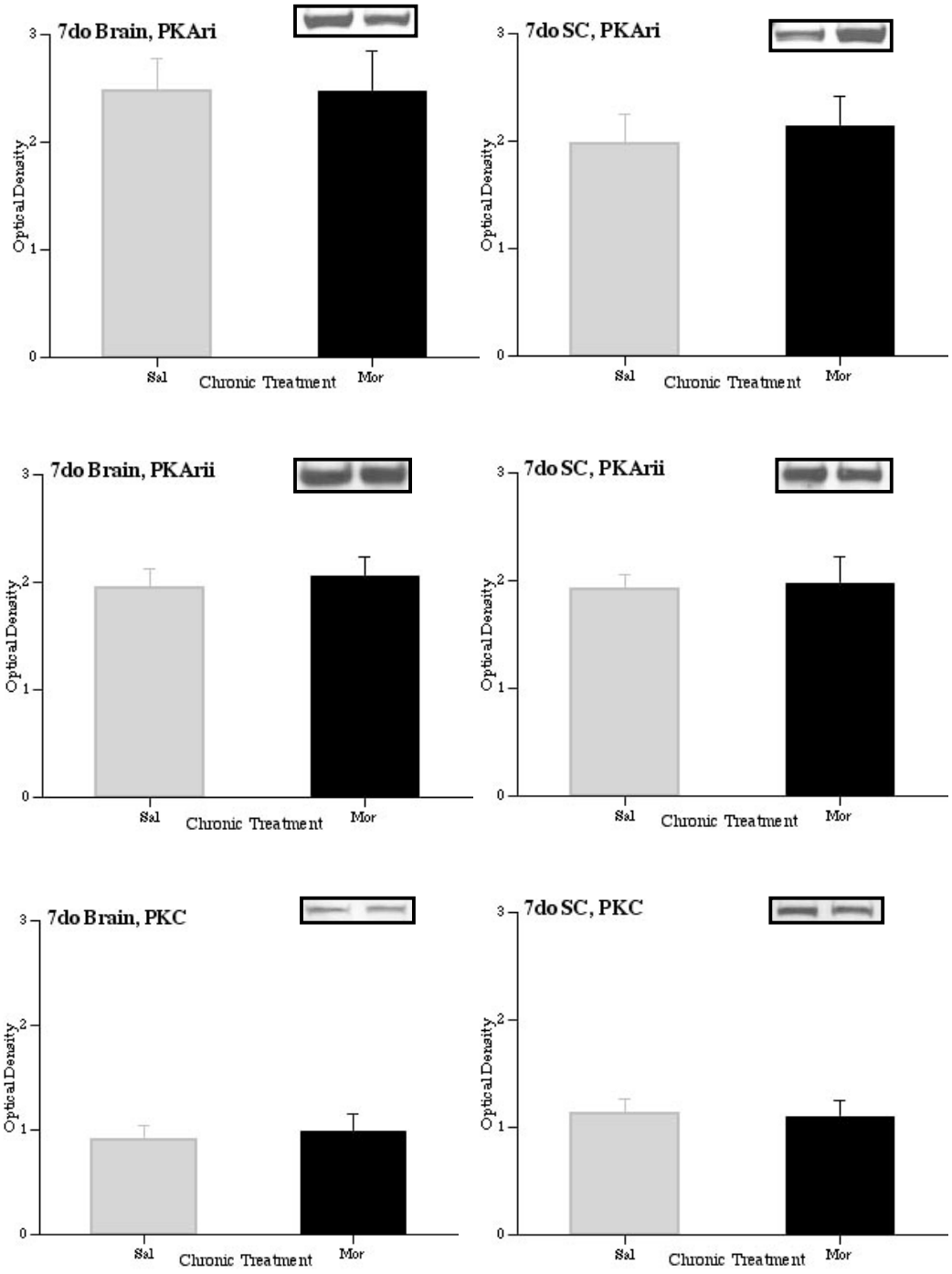


Fig 6-3. The effect of **chronic** morphine treatment on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 7. Animals were treated (ip) with 6.5 daily injections of saline or morphine, two hours after the final injection their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: chronic treatment (10mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 3. Boxes show a representative western blot.

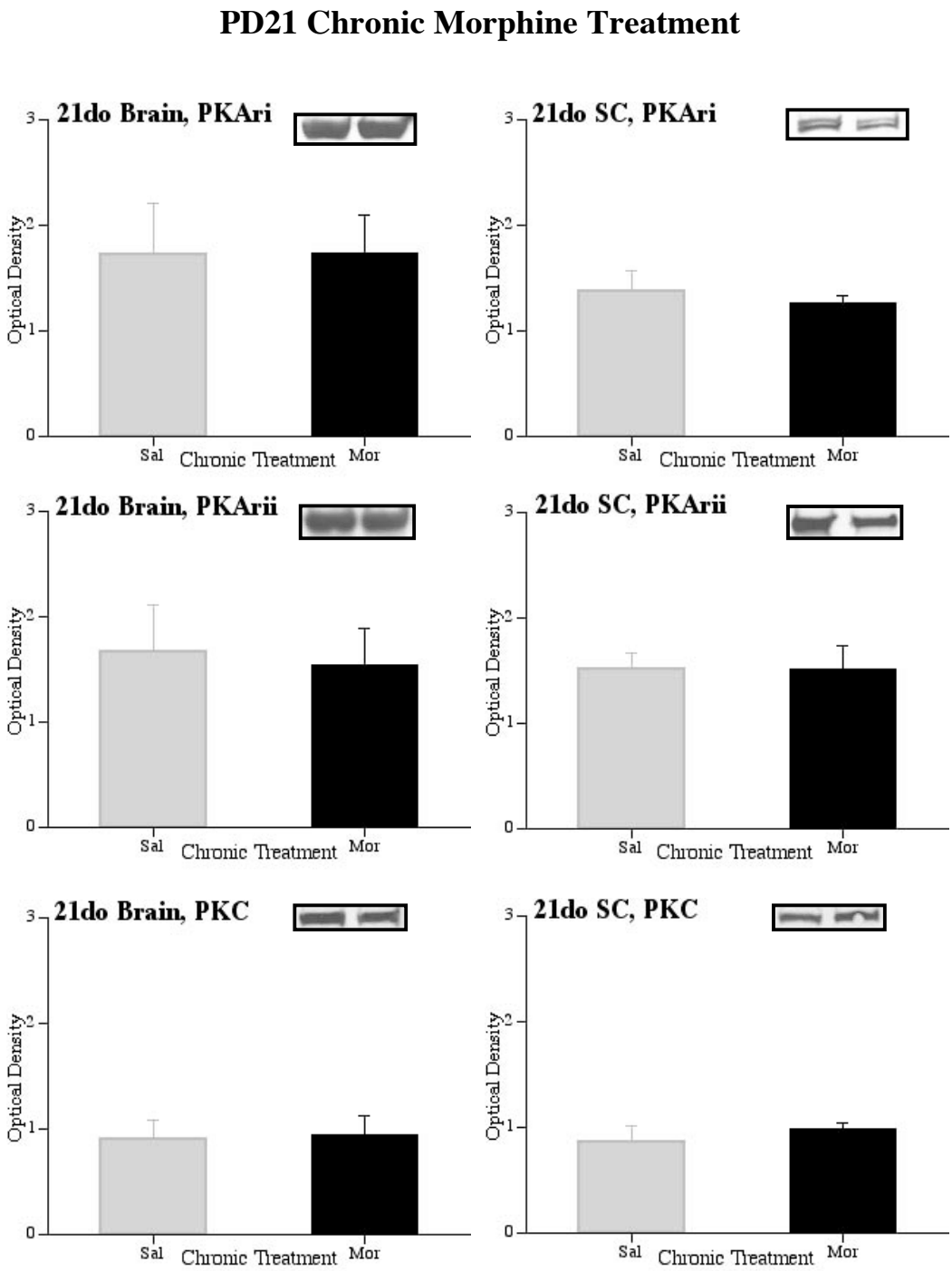


Fig 6-4. The effect of **chronic** morphine treatment on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 21. Animals were treated (ip) with 6.5 daily injections of saline or morphine, two hours after the final injection their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: chronic treatment (10mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 3. Boxes show a representative western blot.

Chronic Morphine Treatment Statistics

Brain

Age/Protein	F	df	P
7do/PKAri	5.0	(1,4)	0.99
7do/PKArii	0.24	(1,4)	0.65
7do/PKC	0.13	(1,4)	0.73
21do/PKAri	0.001	(1,4)	0.98
21do/PKArii	0.04	(1,4)	0.85
21do/PKC	0.01	(1,4)	0.91

Spinal cord

Age/Protein	F	df	P
7do/PKAri	0.16	(1,4)	0.71
7do/PKArii	0.03	(1,4)	0.88
7do/PKC	0.02	(1,4)	0.89
21do/PKAri	0.26	(1,4)	0.64
21do/PKArii	0.001	(1,4)	0.98
21do/PKC	0.58	(1,4)	0.49

Table 6-2. Statistical analysis of **chronic** morphine treatment on levels of each of the PKs at each age group. No significance was found.

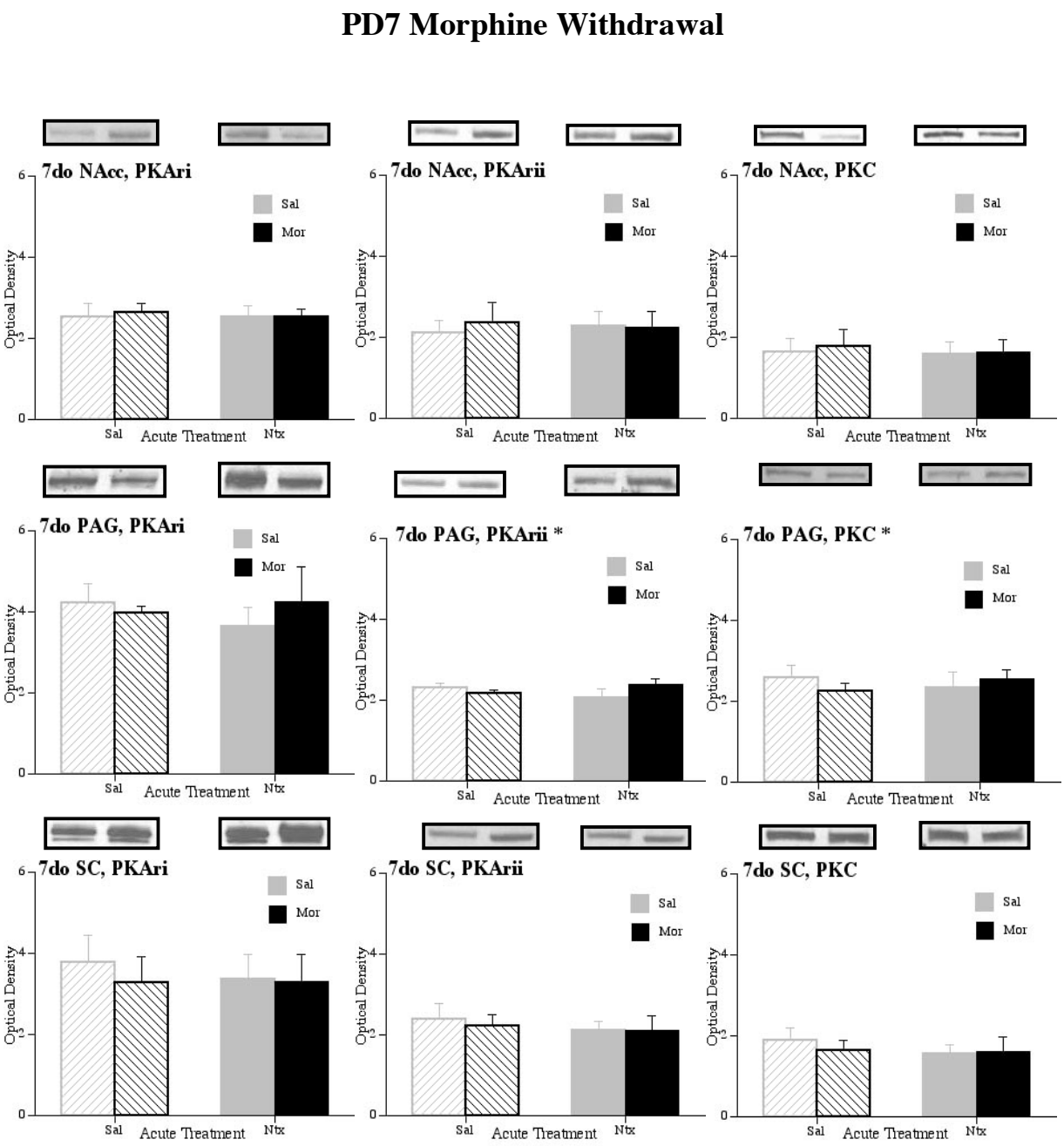


Fig 6-5. The effect of **chronic morphine treatment + precipitated withdrawal** on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 7. Animals were treated (ip) with 6.5 daily injections of saline or morphine, two hours after the final injection they were given an ip injection of saline or naltrexone (to precipitate withdrawal), two hours later their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: acute treatment (1mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 6 (SC & NAcc); N = 3 (PAG). Boxes show a representative western blot. NAcc, nucleus accumbens. PAG, periaqueductal gray area. "*****" Represents a significant effect at p<.05.

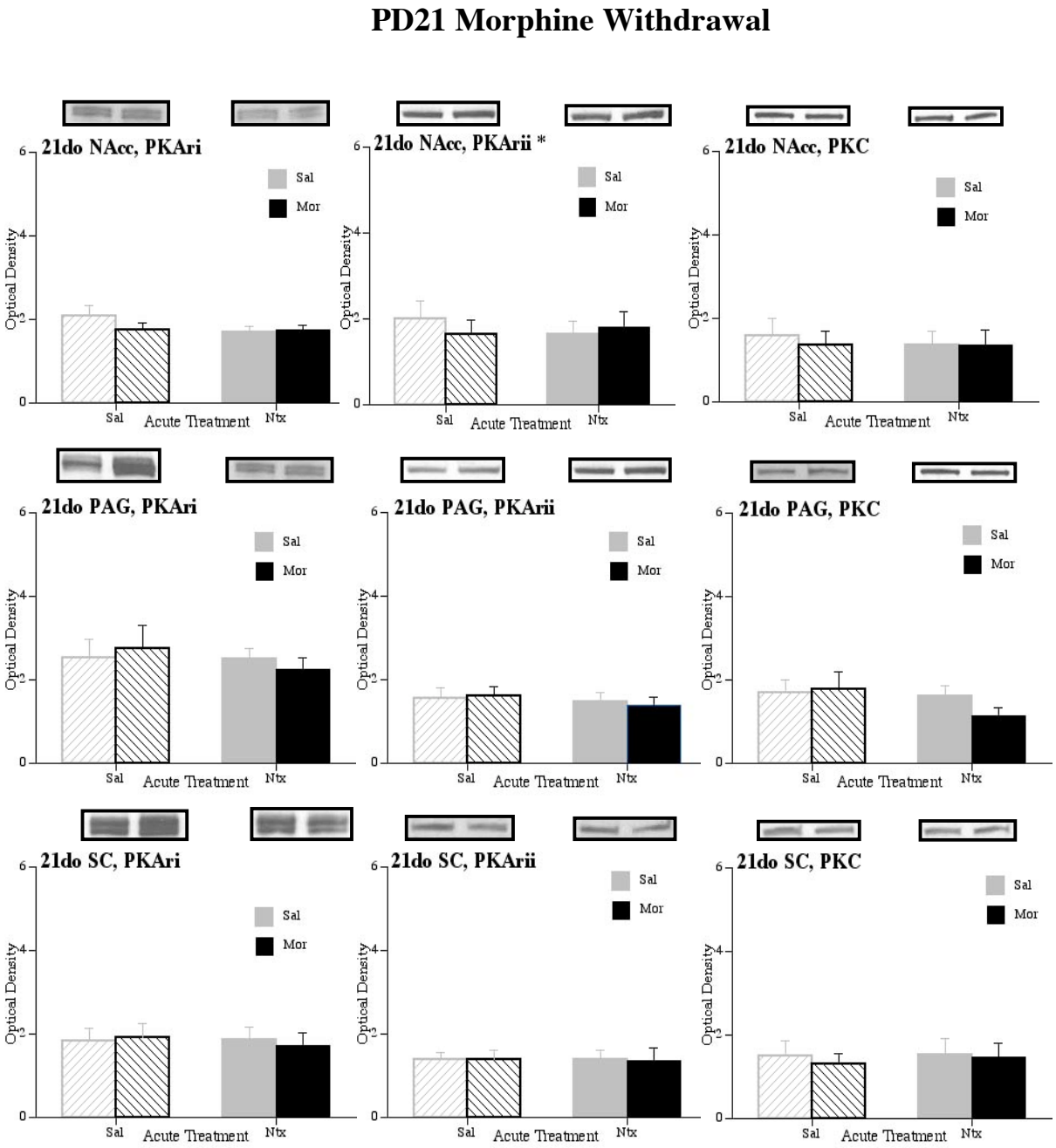


Fig 6-6. The effect of **chronic morphine treatment + precipitated withdrawal** on protein levels of PKC and the two regulatory subunits (ri and rii) of PKA at postnatal day 21. Animals were treated (ip) with 6.5 daily injections of saline or morphine, two hours after the final injection they were given an ip injection of saline or naltrexone (to precipitate withdrawal), two hours later their brains and SCs were removed, and later processed for protein levels using quantitative western analysis. X-axis: acute treatment (1mg/kg). Y-axis: mean optical density (± SEM). Normalized to GAPDH. N = 6 (SC & NAcc); N = 3 (PAG). Boxes show a representative western blot. NAcc, nucleus accumbens. PAG, periaqueductal gray area. "*****" Represents a significant effect at p<.05.

Morphine Withdrawal Statistics

Nucleus Accumbens

Age/Protein	F	df	P
7do/PKAri	0.72	(1,10)	0.42
7do/PKArii	3.63	(1,10)	0.09
7do/PKC	0.22	(1,10)	0.65
21do/PKAri	3.51	(1,10)	0.09
21do/PKArii	10.3	(1,10)	***0.009***
21do/PKC	3.22	(1,10)	0.10

Periaqueductal gray area

Age/Protein	F	df	P
7do/PKAri	0.73	(1,4)	0.44
7do/PKArii	12.5	(1,4)	***0.02***
7do/PKC	13.86	(1,4)	***0.02***
21do/PKAri	1.02	(1,4)	0.37
21do/PKArii	0.19	(1,4)	0.69
21do/PKC	0.6	(1,4)	0.48

Spinal Cord

Age/Protein	F	df	P
7do/PKAri	2.49	(1,10)	0.15
7do/PKArii	0.38	(1,10)	0.55
7do/PKC	1.36	(1,10)	0.27
21do/PKAri	1.56	(1,10)	0.24
21do/PKArii	0.17	(1,10)	0.69
21do/PKC	1.21	(1,10)	0.30

Table 6-3. Statistical analysis of **chronic morphine treatment + precipitated withdrawal** on protein levels of both of the PKs at each age group. Bolded P-values denote significance.

Part Seven: General Discussion

Summary of Overall Findings

Opiate addiction is a devastating problem for both society and the family and friends who interact with the addict. Not only are billions of dollars spent each year on associated problems (Policy 2001) but interpersonal relationships are adversely effected by the individual who has become dependent on them. Early research focused on the behavioral effects of chronic opiate use in human adults and identified comparable effects in human infants. With the advent of rodent models and improved technology, many dynamic and pioneering researchers focused on the cellular and molecular mechanisms, including signal transduction pathways, which regulate opiate tolerance and dependence in an attempt to create drugs with the ability to reverse or reduce the negative side effects of chronic opiate exposure. The areas of focus of these researchers have required the integration of techniques from psychology, behavioral pharmacology, neuroanatomy, genetics, biochemistry, and molecular biology.

The adult rodent literature, from the past two decades, is inundated with studies focusing on all of the major components of the IP_3-DAG and cAMP/adenylyl cyclase signal transduction pathways, but few of these studies have focused on infant rodents. Therefore, the experiments described and analyzed in this dissertation were designed to further explore the underlying mechanisms that regulate opiate tolerance and dependence in the young rodent. To accomplish this, each experiment was designed to closely replicate published adult studies of interest (Cowan, Zhu et al. 1988; Hayward, Duman et al. 1990; Maldonado, Negus et al. 1992b; Nestler, Alreja et al. 1994; Maldonado, Valverde et al. 1995; Narita, Mizoguchi et al. 1995; Chieng, Keay et al. 1995b; Ventayol,

Busquets et al. 1997), with modifications made to accommodate the young rodent. For instance, the established list of adult withdrawal behaviors was modified to include the unique behaviors seen at PD7 to PD21, and pups at PD7 were always returned to their huddles after experimental testing to minimize the distress they typically experience during prolonged isolation.

From these experiments the findings were as follows:

1. *Receptor Mediation of Morphine Dependence*. The μ-opioid receptor plays the predominant role, while the κ- and δ-opioid receptors play a minimal role, in the precipitation of morphine withdrawal at PD7. These results are similar to those found in the adult rodent literature. See table GD1.
2. *Gene Expression During Morphine Dependence*. The precipitation of morphine withdrawal at PD7 is associated with increased levels of Fos-LIR, the protein product of the IEG *c-fos*, in the periaqueductal gray area, spinal cord, locus coeruleus, and nucleus accumbens. These results are similar to those found in the adult rodent literature. See table GD1.
3. *The Protein Kinase and Morphine Interaction*. A) The appearance of morphine withdrawal and tolerance remains unaffected by the acute administration of a broadly acting protein kinase antagonist, a specific PKA antagonist, and a specific PKC antagonist. These results differ from those seen in the adult rodent literature. B) Morphine withdrawal alters levels of PKA and PKC in the PAG at PD7 and PKA in the NAcc at PD21, but neither acute nor chronic morphine treatment alters levels at either age group in the whole brain and spinal cord.

These results both differ and are similar to those seen in the adult rodent literature. See table GD1.

Discussion of Overall Findings

The fact that only activation of μ-ORs precipitates withdrawal at PD7 is consistent with the differential functional role described for each of the opioid receptors in both the infant and adult literature. For instance, at PD10 the administration of a μ- and δ-OR agonist reduces ultrasonic vocalizations (USVs), while a κ-OR agonist increases their occurrence (Carden, Barr et al. 1991). Another study found that at PD5 the acute administration of a μ-OR agonist causes sedation, the administration of a κ-OR agonist causes hyperactivity, while a δ-OR agonist has no behavioral effect (Jackson and Kitchen 1989). In the adult literature the greatest degree of withdrawal is produced by the acute administration of a μ-OR antagonist (Maldonado, Negus et al. 1992b) and the greatest degree of dependence is established by chronic treatment with a μ-OR agonist (Cowan, Zhu et al. 1988). There are well-established studies describing the early gestational appearance of the μ- and κ-ORs, followed by the later appearance of the δ-OR (Kornblum, Hurlbut et al. 1987; De Vries, Hogenboom et al. 1990; Rius, Barg et al. 1991a). All of this combined make a strong argument for the fact that, by birth, the opioid receptors function in a manner comparable to the adult rodent, and therefore are capable of regulating opiate dependence at PD7.

The current findings that c-fos expression increases during morphine withdrawal at PD7 was initially hypothesized. As with the opioid receptors, the expression of the *c-fos* gene during opiate withdrawal is also detectable by early ontogeny (Andersen, LeBlanc et al. 2001; Wiedenmayer and Barr 2001) and is found in brain regions that show activity

in the adult CNS during opiate withdrawal (Jones, Zhu et al. 2002; Zhu, Jenab et al. 2003b). In addition, increased *c-fos* expression is also seen in the hypothalamus and olfactory bulb during PD7 (Maeda, Kishioka et al. 2002) and withdrawal can be precipitated by the microinjection of an opiate antagonist into the locus coeruleus and the periaqueductal gray area (Jones and Barr 2001) in the young rat. All of which is consistent with the adult literature in which Fos-LIR and *c-fos* mRNA are up-regulated in the locus coeruleus, periaqueductal gray area, spinal cord, nucleus accumbens, ventral tegmental area, and amygdala during opiate withdrawal (Hayward, Duman et al. 1990; Chieng, Keay et al. 1995b; Rohde, Detweiler et al. 1996). All of which provides further evidence that the *cfos* gene (and possibly other genes) are functional (and not just detectable) during early ontogeny.

Unlike the findings for the opioid receptors and the *cfos* gene during opiate withdrawal, the results from PKA and PKC do not support the hypothesis that they play a significant role in opiate tolerance and dependence in the young rat. Blocking their activity with specific and broadly acting protein kinase antagonists does not reduce/block the expression of morphine tolerance and dependence at PD7 or 21. In addition, overall, acute and chronic morphine treatment, as well as precipitated withdrawal have no effect on protein levels of PKA and PKC. This is in contrast to the adult literature where its has been found that acute blockade of PKA or PKC activity attenuates opiate tolerance and dependence (Maldonado, Valverde et al. 1995; Granados-Soto, Kalcheva et al. 2000; Javed, Dewey et al. 2004), and the different morphine treatment paradigms exert an effect on the protein and activity levels of both PKA and PKC (Ventayol, Busquets et al. 1997; Bernstein and Welch 1998; Harlan, Kailas et al. 2004).

In addition, developmental studies have found that G-proteins, adenylyl cyclase, cAMP, and PKC are detectable by birth. See table 2-2. One possible explanation for the contradictory findings of the current experiments is that detectability does not equate to functionality. Such that the presence of the protein kinases (and the second messengers that activate them) does not necessarily mean that they will function. This has been found to be the case with the μ-OR, in which young rats are more sensitive to the antinociceptive effects of morphine (as compared to fully mature adults), despite the fact that the μ-OR shows weaker coupling to their G-proteins (Windh and Kuhn 1995a).

Future Directions

The current experiments were meant as basic animal models to help researchers further understand the human infant system and one day successfully design drugs that can be tailor made to prevent or treat infants born addicted to opiates. While the information gleaned from these experiments are interesting, they are by no means assumed to paint a complete picture of the interaction between signal transduction pathways and the opiate system. Many more infant experiments, using multiple paradigms, must be conducted to catch up to the knowledge researchers have about adult rodents and the opiate-signal transduction pathway interaction. Therefore this section suggests future directions that should be explored as they relate to the experiments presented in this dissertation, in an effort to accurately understand the way in which the infant CNS functions after long-term exposure to opiates.

Although *opioid receptors* do not play the sole modulatory role in the long-term effects of opiates the activation of any intracellular signal transduction pathway begins with the binding of the exogenous substance to the receptor and the initial rate and duration of the

intracellular affect is determined by the ligand/receptor interaction (Kandel, Schwartz et al. 2000). Work has been done on opioid receptors in the young rodent (Barr, Paredes et al. 1986; Carden, Barr et al. 1991; Barr, Miya et al. 1992b; Barr and Wang 1994a; Barr, Wang et al. 1994b), but it would be interesting to further explore the exact role that each receptor plays in conditioned place aversion/preference, tolerance, and ultrasonic vocalizations throughout ontogeny (which includes gestation, PD7, PD14, and PD21). In the adult literature there is evidence that the different opioid receptor classes have the ability to regulate each other, when they become active. For instance, DAergic neuronal firing in the ventral tegmental area, which was previously shown to increase with treatment of a μ-OR agonist, was later shown to be inhibited by treatment with a κ-OR agonist (Margolis, Hjelmstad et al. 2003). Therefore one could explore the role of the δ-OR in this paradigm, as well as other paradigms with opioid receptor interactions during early ontogeny.

Describing and understanding how *genes* function has fascinated geneticist since the time of Mendel and current issues surrounding genes have been met with fascination (eg., the popularization of "genes for" schizophrenia or depression and the creation of transgenic mice), as well as fear (eg., apprehension over cloning human babies and genetic screening by insurance companies). At the heart of it all is the fact that genes play a critical role in translating the acute cellular changes from the internal and external environment into long-term alterations (Nestler 2001), making them important in understanding the long-term adaptations that result from opiate exposure. In addition to *c-fos*, there are numerous additional IEG genes like *c-jun* and *zif*/268 (Hayward, Duman et al. 1990; Sheng and Greenberg 1990; Beckmann, Matsumoto et al. 1995), as well as

late response genes and other gene categories that include CREB, the nerve growth factor gene, and the tyrosine hydroxylase gene (Guitart, Hayward et al. 1990; Sheng and Greenberg 1990; Nestler 1992; Lane-Ladd, Pineda et al. 1997) that have been studied in the adult model of tolerance and dependence. Since genes are so crucial to cellular function and adaptation, it would be important to study these genes in the young rodent during withdrawal, as well as during acute and chronic treatment (Nestler 2001). In addition, there are many other brain regions that show involvement in opiate tolerance and dependence and need to be explored in the infant rodent, like the VTA, amygdala, frontal cortex, nucleus raphe magnus, and hippocampus (Kimes and London 1989; Hayward, Duman et al. 1990; Busquets, Escriba et al. 1995; Chieng, Keay et al. 1995b; Rodriguez Parkitna, Bilecki et al. 2004). See figure 2-3. For instance, Ikemoto (Ikemoto, Inoue et al. 2002) identified a protein in the mouse amygdala, designated "addicsin", that is up-regulated by chronic morphine and contains two possible sites for phosphorylation by PKC. Within the nucleus raphe magnus the increased release of glutamate from μ-OR containing secondary neurons is blocked by the administration of an adenylyl cyclase, PKA, or PKC antagonist (Bie and Pan 2005).

As previously mentioned, the phosphorylation and dephosphorylation of target proteins is a crucial step in normal neuronal functioning and communication (Liu and Anand 2001; Moncada, Cendan et al. 2003). *Protein kinases* are important for their role in protein phosphorylation (Liu, Zhang et al. 2002; Moncada, Cendan et al. 2003). In addition to PKA and PKC, there are numerous other pathways and their related protein kinases that interact with the opiate system in the adult rodent (Liu and Anand 2001). The activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) potentiates the

expression of opiate tolerance (Mestek, Hurley et al. 1995) and acute morphine treatment increases CaMKII activity, while chronic morphine treatment reduces its activity in the rat hippocampus (Lou, Zhou et al. 1999).

Blocking mitogen-activated protein kinase (MAPK) activity reduces the expression of opiate tolerance in the mouse dorsal root ganglion (Tan, Groszer et al. 2003) and acute morphine treatment results in modulation (both increased and decreased) of its activity in multiple brain regions, including the LC, amygdala, and NAcc (Eitan, Bryant et al. 2003). For the protein kinase G protein-coupled receptor kinase (GRK), acute morphine treatment increases its levels while chronic morphine has no effect (Fan, Zhang et al. 2002). Finally, in the NAcc levels of phosphorylated extracellular signal-regulated protein kinase (pERK) are increased by acute morphine treatment and unaffected by chronic morphine treatment (Muller and Unterwald 2004), although in the cerebral cortex chronic treatment causes downregulation (Ferrer-Alcon, M et al. 2004). The way in which these protein kinases interact with the opiate system is an area that is largely unexplored and would provide invaluable information on molecular mechanisms during development, making them ideal to study in the young rodent.

Protein phosphatases (PPs) are proteins that have the ability to remove a phosphate group and thus reverse the phosphorylating effects of protein kinases (Alberts, Bray et al. 1994). In this capacity, the interaction between protein kinases and protein phosphatases are just as important to the long-term effects of opiates, as protein kinases alone (Bernstein and Welch 1998; Moncada, Cendan et al. 2003). To date, there are no published studies looking at protein phosphatases and their interaction with the opiate system in the young rodent, making phosphatases another crucial area to investigate.

In adult mice, the acute blockade of PP2A, but not PP1, reduces the expression of acute morphine-induced antinociception (Moncada, Cendan et al. 2003). While blocking the activity of both PPs in tolerant mice enhances morphine-induced antinociception (Bernstein and Welch 1998). Finally, KEPI, a gene that blocks PP1 activity and is phosphorylated by PKC, is up-regulated in adult mice and rat brains by acute and chronic morphine treatment (Liu, Zhang et al. 2002). These findings in the adult literature should lead the way for related studies in the infant rodent, in order to fully comprehend the mechanisms that underlie opiate tolerance and dependence during early ontogeny.

The issue of opiate dependence and tolerance is just as important for human infants as it is for human adults. For this reason, the overall aim of this dissertation was to use an animal model to better understand the relationship between signal transduction systems (IP_3-DAG and cAMP/adenylyl cyclase) and the opiate system during the development of the CNS. Although not expected to be accomplished by the experiments described in the previous chapters, the long-term goal is to determine appropriate therapies to prevent or reduce opiate tolerance and dependence in the human infant. One such study has found that chronic low levels of the opiate antagonist naltrexone have the ability to reduce the expression of phosphorylated CREB, PKArii, and *cfos* in the nucleus tractus solitarus and the LC (Mannelli, Gottheil et al. 2004), in adult rats. Promising therapies like this may be effective in young humans, but in order for them to be successful they must first be tested in the developing animal rodent model.

Similar/Different Mechanisms Throughout Development

Mechanisms in common (active throughout ontogeny)

1. The μ -OR receptor plays the primary role in the expression of precipitated withdrawal behaviors at PD7. See chapter 3.

2. The *cfos* gene is active during precipitated morphine withdrawal in the PAG, NAcc, SC, and LC at PD7. See chapter 4.

3. Acute precipitated morphine withdrawal alters protein levels of PKArii and PKC in the PAG at PD7 and PKArii in the NAcc at PD21. See chapter 6.

Mechanisms that differ (active only in adult rodents)

1. The acute blockade of PKA and PKC does not reduce/block precipitated morphine withdrawal and tolerance at PD7 and PD21. See chapter 5.

2. Acute morphine treatment does not reduce levels of PKA or PKC at either PD7 or PD21. See chapter 6.

3. Chronic morphine treatment does not effect levels of PKA or PKC at either PD7 or PD21. See chapter 6.

4. Acute precipitated morphine withdrawal does not alter levels of PKA and PKC in the NAcc and SC or levels of PKAri in the PAG at PD7. The same treatment does not alter levels of PKA and PKC in the PAG and SC or PKAri and PKC in the NAcc at PD21. See chapter 6.

Table GD-1. An outline of the mechanisms that, based on the findings of the present dissertation, have been shown to be involved in opiate tolerance and dependence in both the adult rodent and infant rat, or only involved in the adult rodent.

References

Abdelhamid, E. E., M. Sultana, et al. (1991). Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. *J Pharmacol Exp Ther* 258(1): 299-303.

Adams, R. E. and G. F. Wooten (1990). Dependence and withdrawal following intracerebroventricular and systemic morphine administration: functional anatomy and behavior. *Brain Res* 518(1-2): 6-10.

Aghajanian, G. K. (1978). Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature 276(5684): 186-8.

Alberts, B., D. Bray, et al. (1994). *Molecular biology of the cell*. New York, Garland Publishing.

Aley, K. O. and J. D. Levine (1997b). Different mechanisms mediate development and expression of tolerance and dependence for peripheral mu-opioid antinociception in rat. *J Neurosci* 17(20): 8018-23.

Ammon, S., P. Mayer, et al. (2003). Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Brain Res Mol Brain Res* 112(1-2): 113-25.

Anand, K. J. and J. H. Arnold (1994). Opioid tolerance and dependence in infants and children. *Crit Care Med* 22(2): 334-42.

Andersen, S. L., C. J. LeBlanc, et al. (2001). Maturational increases in c-fos expression in the ascending dopamine systems. *Synapse* 41(4): 345-50.

Andrade, R., C. P. Vandermaelen, et al. (1983). Morphine tolerance and dependence in the locus coeruleus: single cell studies in brain slices. *Eur J Pharmacol* 91(2-3): 161-9.

Aston-Jones, G., H. Hirata, et al. (1997). Local opiate withdrawal in locus coeruleus in vivo. *Brain Res* 765(2): 331-6.

Badiani, A., P. Leone, et al. (1995). Intra-VTA injections of the mu-opioid antagonist CTOP enhance locomotor activity. *Brain Res* 690(1): 112-6.

Bagley, E. E., M. B. Gerke, et al. (2005). GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. *Neuron* 45(3): 433-45.

Bandler, R. and M. T. Shipley (1994). Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci* 17(9): 379-89.

Barr, G. A. and G. A. Goodwin (1997). Precipitated morphine withdrawal induces a conditioned aversion in the preweaning rat. *Pharmacol Biochem Behav* 57(4): 779-83.

Barr, G. A., D. Y. Miya, et al. (1992b). Analgesic effects of intraventricular and intrathecal injection of morphine and ketocyclazocine in the infant rat. *Brain Res* 584(1-2): 83-91.

Barr, G. A., W. Paredes, et al. (1986). Kappa opioid receptor-mediated analgesia in the developing rat. *Brain Res* 394(2): 145-52.

Barr, G. A. and G. Rossi (1992c). Conditioned place preference from ventral tegmental injection of morphine in neonatal rats. *Brain Res Dev Brain Res* 66(1): 133-6.

Barr, G. A. and S. Wang (1992d). Tolerance and withdrawal to chronic morphine treatment in the week-old rat pup. *Eur J Pharmacol* 215(1): 35-42.

Barr, G. A. and S. Wang (1994a). Mu and delta opiates induce analgesia when injected into the PAG or NRM in preweanling rats. *Regulatory peptides* 54: 17-8.

Barr, G. A., S. Wang, et al. (1994b). Aversive properties of the kappa opioid agonist U50,488 in the week-old rat pup. *Psychopharmacology* (Berl) 113(3-4): 422-8.

Battaini, F., R. Del Vesco, et al. (1990). Regulation of phorbol ester binding and protein kinase C activity in aged rat brain. *Neurobiol Aging* 11(5): 563-6.

Battaini, F., S. Elkabes, et al. (1995). Protein kinase C activity, translocation, and conventional isoforms in aging rat brain. *Neurobiol Aging* 16(2): 137-48.

Bear, M. F., B. W. Connors, et al. (2001). Neuroscience; exploring the brain. Baltimore, Lippincott Williams & Wilkins.

Beckmann, A. M., I. Matsumoto, et al. (1995). Immediate early gene expression during morphine withdrawal. *Neuropharmacology* 34(9): 1183-9.

Belanger, S., W. Ma, et al. (2002). Expression of calcitonin gene-related peptide, substance P and protein kinase C in cultured dorsal root ganglion neurons following chronic exposure to mu, delta and kappa opiates. *Neuroscience* 115(2): 441-53.

Belcheva, M. M., L. M. Bohn, et al. (1998). Brain opioid receptor adaptation and expression after prenatal exposure to buprenorphine. *Brain Res Dev Brain Res* 111(1): 35-42.

Benavides, M., M. L. Laorden, et al. (2003). Regulation of tyrosine hydroxylase levels and activity and Fos expression during opioid withdrawal in the hypothalamic PVN and medulla oblongata catecholaminergic cell groups innervating the PVN. *Eur J Neurosci* 17(1): 103-12.

Bernstein, M. A. and S. P. Welch (1995). Alterations in L-type calcium channels in the brain and spinal cord of acutely treated and morphine-tolerant mice. *Brain Res* 696(1-2): 83-8.

Bernstein, M. A. and S. P. Welch (1997). Effects of spinal versus supraspinal administration of cyclic nucleotide-dependent protein kinase inhibitors on morphine tolerance in mice. *Drug Alcohol Depend* 44(1): 41-6.

Bernstein, M. A. and S. P. Welch (1998a). Inhibition of protein phosphatases alters the expression of morphine tolerance in mice. *Eur J Pharmacol* 341(2-3): 173-7.

Bernstein, M. A. and S. P. Welch (1998b). mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Brain Res Mol Brain Res* 55(2): 237-42.

Besunder, J. B., M. D. Reed, et al. (1988). Principles of drug biodisposition in the neonate. A critical evaluation of the pharmacokinetic-pharmacodynamic interface (Part I). *Clin Pharmacokinet* 14(4): 189-216.

Bhargava, H. N. and Y. J. Cao (1997). Effect of chronic administration of morphine, U-50, 488H and [D-Pen2, D-Pen5]enkephalin on the concentration of cGMP in brain regions and spinal cord of the mouse. *Peptides* 18(10): 1629-34.

Bhargava, H. N. and A. Gulati (1990). Down-regulation of brain and spinal cord mu-opiate receptors in morphine tolerant-dependent rats. *Eur J Pharmacol* 190(3): 305-11.

Bie, B. and Z. Z. Pan (2005). Increased glutamate synaptic transmission in the nucleus raphe magnus neurons from morphine-tolerant rats. *Mol Pain* 1(1): 7.

Bilsky, E. J., R. N. Bernstein, et al. (1996). Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. *J Pharmacol Exp Ther* 277(1): 484-90.

Bird, S. J. and M. J. Kuhar (1977). Iontophoretic application of opiates to the locus coeruleus. *Brain Res* 122(3): 523-33.

Blendy, J. A. and R. Maldonado (1998). Genetic analysis of drug addiction: the role of cAMP response element binding protein. *J Mol Med* 76(2): 104-10.

Blume, A. J., D. Lichtshtein, et al. (1979). Coupling of opiate receptors to adenylate cyclase: requirement for Na+ and GTP. *Proc Natl Acad Sci U S A* 76(11): 5626-30.

Bläsig, J., A. Herz, et al. (1973). Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. *Psychopharmacologia* 33(1): 19-38.

Boucher, T., E. Jennings, et al. (1998). The onset of diffuse noxious inhibitory controls in postnatal rat pups: a C-Fos study. *Neurosci Lett* 257(1): 9-12.

Bozarth, M. A. and R. A. Wise (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science* 224(4648): 516-7.

Brady, L. S., M. Herkenham, et al. (1989). Chronic morphine increases mu-opiate receptor binding in rat brain: a quantitative autoradiographic study. *Brain Res* 477(1-2): 382-6.

Brodsky, M., K. Elliott, et al. (1995). CNS levels of mu opioid receptor (MOR-1) mRNA during chronic treatment with morphine or naltrexone. *Brain Res Bull* 38(2): 135-41.

Buckett, W. R. (1964). A new test for morphine-like physical dependence (addiction liability) in rats. *Psychopharmacologia* 6(6): 410-6.

Busquets, X., P. V. Escriba, et al. (1995). Loss of protein kinase C-alpha beta in brain of heroin addicts and morphine-dependent rats. *J Neurochem* 64(1): 247-52.

Buzas, B., J. Rosenberger, et al. (1996). Mu and delta opioid receptor gene expression after chronic treatment with opioid agonist. *Neuroreport* 7(9): 1505-8.

Cadoni, C. and G. Di Chiara (1999). Reciprocal changes in dopamine responsiveness in the nucleus accumbens shell and core and in the dorsal caudate-putamen in rats sensitized to morphine. *Neuroscience* 90(2): 447-55.

Calvino, B., J. Lagowska, et al. (1979). Morphine withdrawal syndrome: differential participation of structures located within the amygdaloid complex and striatum of the rat. *Brain Res* 177(1): 19-34.

Carden, S. E., G. A. Barr, et al. (1991). Differential effects of specific opioid receptor agonists on rat pup isolation calls. *Brain Res Dev Brain Res* 62(1): 17-22.

Castelli, M. P., M. Melis, et al. (1997). Chronic morphine and naltrexone fail to modify mu-opioid receptor mRNA levels in the rat brain. *Brain Res Mol Brain Res* 45(1): 149-53.

Cerezo, M., M. L. Laorden, et al. (2002). Inhibition of protein kinase C but not protein kinase A attenuates morphine withdrawal excitation of rat hypothalamus-pituitary-adrenal axis. *Eur J Pharmacol* 452(1): 57-66.

Chakrabarti, S. and A. R. Gintzler (2003a). Phosphorylation of Gbeta is augmented by chronic morphine and enhances Gbetagamma stimulation of adenylyl cyclase activity. *Brain Res Mol Brain Res* 119(2): 144-51.

Chakrabarti, S., P. Y. Law, et al. (1995). Neuroblastoma Neuro2A cells stably expressing a cloned mu-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Brain Res Mol Brain Res* 30(2): 269-78.

Chakrabarti, S., P. Y. Law, et al. (1998a). Distinct differences between morphine- and [D-Ala2,N-MePhe4,Gly-ol5]-enkephalin-mu-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. *J Neurochem* 71(1): 231-9.

Chakrabarti, S., N. J. Liu, et al. (2003b). Reciprocal modulation of phospholipase Cbeta isoforms: adaptation to chronic morphine. *Proc Natl Acad Sci U S A* 100(23): 13686-91.

Chakrabarti, S., A. Regec, et al. (2005). Chronic morphine acts via a protein kinase Cgamma-G(beta)-adenylyl cyclase complex to augment phosphorylation of G(beta) and G(betagamma) stimulatory adenylyl cyclase signaling. *Brain Res Mol Brain Res*. In press.

Chakrabarti, S., L. Wang, et al. (1998c). Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence. *Mol Pharmacol* 54(6): 949-53.

Chang, S. L., S. P. Squinto, et al. (1988). Morphine activation of c-fos expression in rat brain. *Biochem Biophys Res Commun* 157(2): 698-704.

Chartoff, E. H., M. Papadopoulou, et al. (2003). Dopamine-dependent increases in phosphorylation of cAMP response element binding protein (CREB) during precipitated morphine withdrawal in primary cultures of rat striatum. *J Neurochem* 87(1): 107-18.

Chen, Y. and L. Yu (1994). Differential regulation by cAMP-dependent protein kinase and protein kinase C of the mu opioid receptor coupling to a G protein-activated K+ channel. *J Biol Chem* 269(11): 7839-42.

Chieng, B., K. A. Keay, et al. (1995b). Increased fos-like immunoreactivity in the periaqueductal gray of anaesthetised rats during opiate withdrawal. *Neurosci Lett* 183(1-2): 79-82.

Chieng, B. and J. T. Williams (1998). Increased opioid inhibition of GABA release in nucleus accumbens during morphine withdrawal. *J Neurosci* 18(17): 7033-9.

Childers, S. R. (1991). Opioid receptor-coupled second messenger systems. *Life Sci* 48(21): 1991-2003.

Christie, M. J., J. T. Williams, et al. (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Mol Pharmacol* 32(5): 633-8.

Christie, M. J., J. T. Williams, et al. (1997). Where is the locus in opioid withdrawal? *Trends Pharmacol Sci* 18(4): 134-40.

Collier, H. O. (1965). A General Theory of the Genesis of Drug Dependence by Induction of Receptors. *Nature* 205: 181-2.

Collin, E. and F. Cesselin (1991). Neurobiological mechanisms of opioid tolerance and dependence. *Clin Neuropharmacol* 14(6): 465-88.

Collins, R. (1985). Atlas of drug reactions. New York, Churchill Livingstone.

Cooper, J., F. E. Bloom, et al. (1996). The biochemical basis of neuropharmacology. New York, Oxford University Press.

Cowan, A., X. Z. Zhu, et al. (1988). Direct dependence studies in rats with agents selective for different types of opioid receptor. *J Pharmacol Exp Ther* 246(3): 950-5.

Cruciani, R. A., B. Dvorkin, et al. (1993). Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proc Natl Acad Sci U S A* 90(7): 3019-23.

Da Costa Gomez, T. M., S. D. Chandler, et al. (1996). The role of the basolateral nucleus of the amygdala in the pathway between the amygdala and the midbrain periaqueductal gray in the rat. *Neurosci Lett* 214(1): 5-8.

Dalton, G. D., F. L. Smith, et al. (2005). Alterations in brain Protein Kinase A activity and reversal of morphine tolerance by two fragments of native Protein Kinase A inhibitor peptide (PKI). *Neuropharmacology* 48(5): 648-57.

Dang, V. C. and J. T. Williams (2004). Chronic morphine treatment reduces recovery from opioid desensitization. J Neurosci 24(35): 7699-706.

Danks, J. A., F. C. Tortella, et al. (1988). Chronic administration of morphine and naltrexone up-regulate[3H][D-Ala2,D-leu5]enkephalin binding sites by different mechanisms. *Neuropharmacology* 27(9): 965-74.

De Vries, T. J., F. Hogenboom, et al. (1990). Ontogeny of mu-, delta- and kappa-opioid receptors mediating inhibition of neurotransmitter release and adenylate cyclase activity in rat brain. *Brain Res Dev Brain Res* 54(1): 63-9.

De Vries, T. J., B. J. Van Vliet, et al. (1991). Effect of chronic prenatal morphine treatment of mu-opioid receptor-regulated adenylate cyclase activity and neurotransmitter release in rat brain slices. *Eur J Pharmacol* 208(2): 97-104.

Delander, G. E. and A. E. Takemori (1983). Spinal antagonism of tolerance and dependence induced by systemically administered morphine. *Eur J Pharmacol* 94(1-2): 35-42.

Di Chiara, G. and R. A. North (1992). Neurobiology of opiate abuse. *Trends Pharmacol Sci* 13(5): 185-93.

Diana, M., M. Pistis, et al. (1995). Profound decrease of mesolimbic dopaminergic neuronal activity in morphine withdrawn rats. *J Pharmacol Exp Ther* 272(2): 781-5.

Dinges, D. F., M. M. Davis, et al. (1980). Fetal exposure to narcotics: neonatal sleep as a measure of nervous system disturbance. *Science* 209(4456): 619-21.

Duman, R. S., J. F. Tallman, et al. (1988). Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J Pharmacol Exp Ther* 246(3): 1033-9.

Dworkin, S. I., G. F. Guerin, et al. (1988). Lack of an effect of 6-hydroxydopamine lesions of the nucleus accumbens on intravenous morphine self-administration. *Pharmacol Biochem Behav* 30(4): 1051-7.

Eddy, N. B., H. Halbach, et al. (1965). Drug dependence: its significance and characteristics. Bull World Health Organ 32(5): 721-33.

Eitan, S., C. D. Bryant, et al. (2003). Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci* 23(23): 8360-9.

Erdtmann-Vourliotis, M., P. Mayer, et al. (1998). Identification of brain regions that are markedly activated by morphine in tolerant but not in naive rats. *Brain Res Mol Brain Res* 61(1-2): 51-61.

Escriba, P. V., M. Sastre, et al. (1994). Increased density of guanine nucleotide-binding proteins in the postmortem brains of heroin addicts. *Arch Gen Psychiatry* 51(6): 494-501.

Fan, X., J. Zhang, et al. (2002). Acute and chronic morphine treatments and morphine withdrawal differentially regulate GRK2 and GRK5 gene expression in rat brain. *Neuropharmacology* 43(5): 809-16.

Fanselow, M. S. and C. P. Cramer (1988). The ontogeny of opiate tolerance and withdrawal in infant rats. *Pharmacol Biochem Behav* 31(2): 431-8.

Feldman, R. S., J. S. Meyer, et al. (1997). Principles of neuropsychopharmacology. MA, Sinauer Associates, Inc.

Ferrer-Alcon, M., J. G.-F. M, et al. (2004). Long-term regulation of signalling components of adenylyl cyclase and mitogen-activated protein kinase in the pre-frontal cortex of human opiate addicts. *J Neurochem* 90(1): 220-30.

Finnegan, L. P. (1985). Effects of maternal opiate abuse on the newborn. *Fed Proc* 44(7): 2314-7.

Fleming, L. M., G. Ponjee, et al. (1992). Inhibition of protein phosphorylation by opioid-inhibited adenylyl cyclase in rat brain membranes. *J Pharmacol Exp Ther* 260(3): 1416-24.

Foote, S. L., F. E. Bloom, et al. (1983). Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. *Physiol Rev* 63(3): 844-914.

Franck, L. S. and C. Miaskowski (1998). The use of intravenous opioids to provide analgesia in critically ill, premature neonates: a research critique. *J Pain Symptom Manage* 15(1): 41-69.

Frankel, P. S., R. E. Harlan, et al. (1999a). Chronic administration of morphine alters immediate-early gene expression in the forebrain of post-dependent rats. *Brain Res* 835(2): 204-12.

Freedman, J. E. and G. K. Aghajanian (1985). Opiate and alpha 2-adrenoceptor responses of rat amygdaloid neurons: co- localization and interactions during withdrawal. *J Neurosci* 5(11): 3016-24.

Frenois, F., M. Cador, et al. (2002). Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* 16(7): 1377-89.

Fundytus, M. E. and T. J. Coderre (1996). Chronic inhibition of intracellular Ca2+ release or protein kinase C activation significantly reduces the development of morphine dependence. *Eur J Pharmacol* 300(3): 173-81.

Fundytus, M. E. and T. J. Coderre (1999). Opioid tolerance and dependence. *Pain Forum* 8(1): 3-13.

Garcia, M. M., H. E. Brown, et al. (1995). Alterations in immediate-early gene proteins in the rat forebrain induced by acute morphine injection. *Brain Res* 692(1-2): 23-40.

Garcia-Sevilla, J. A., P. Ventayol, et al. (1997). Regulation of immunolabelled mu-opioid receptors and protein kinase C- alpha and zeta isoforms in the frontal cortex of human opiate addicts. *Neurosci Lett* 226(1): 29-32.

Geary, W. A., 2nd and G. F. Wooten (1985). Dose effects of naloxone on fixed morphine dependence: simultaneous behavioral and 2-deoxyglucose study in the rat. *Brain Res* 332(1): 69-78.

Geller, L. M. and E. H. Geller (1966). A simple technique for the permanent marking of newborn albino rats. *Psychol Rep* 18(1): 221-2.

Georges, F., E. Normand, et al. (1998). Opioid receptor gene expression in the rat brain during ontogeny, with special reference to the mesostriatal system: an in situ hybridization study. *Brain Res Dev Brain Res* 109(2): 187-99.

Georges, F., L. Stinus, et al. (2000). Mapping of c-fos gene expression in the brain during morphine dependence and precipitated withdrawal, and phenotypic identification of the striatal neurons involved. *Eur J Neurosci* 12(12): 4475-86.

Gmerek, D. E., L. A. Dykstra, et al. (1987). Kappa opioids in rhesus monkeys. III. Dependence associated with chronic administration. *J Pharmacol Exp Ther* 242(2): 428-36.

Gold, L. H., L. Stinus, et al. (1994). Prolonged tolerance, dependence and abstinence following subcutaneous morphine pellet implantation in the rat. *Eur J Pharmacol* 253(1-2): 45-51.

Gracy, K. N., L. A. Dankiewicz, et al. (2001). Opiate withdrawal-induced fos immunoreactivity in the rat extended amygdala parallels the development of conditioned place aversion. *Neuropsychopharmacology* 24(2): 152-60.

Granados-Soto, V., I. Kalcheva, et al. (2000). Spinal PKC activity and expression: role in tolerance produced by continuous spinal morphine infusion. *Pain* 85(3): 395-404.

Grilly, D. M. (1998). Drugs and human behavior. Boston, Allyn and Bacon.

Guitart, X., M. Hayward, et al. (1990). Identification of MARPP-58, a morphine- and cyclic AMP-regulated phosphoprotein of 58 kDa, as tyrosine hydroxylase: evidence for regulation of its expression by chronic morphine in the rat locus coeruleus. *J Neurosci* 10(8): 2649-59.

Guitart, X. and E. J. Nestler (1993). Second messenger and protein phosphorylation mechanisms underlying opiate addiction: studies in the rat locus coeruleus. *Neurochem Res* 18(1): 5-13.

Guitart, X., M. A. Thompson, et al. (1992). Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. J Neurochem 58(3): 1168-71.

Hakan, R. L., C. Callaway, et al. (1989a). Electrophysiological analysis of the neural circuitry underlying opiate effects in the nucleus accumbens septi. *Neurosci Lett* 101(2): 163-8.

Hakan, R. L. and S. J. Henriksen (1989b). Opiate influences on nucleus accumbens neuronal electrophysiology: dopamine and non-dopamine mechanisms. *J Neurosci* 9(10): 3538-46.

Han, S. H., Y. W. Cho, et al. (1999). Mu-opioid agonist-induced activation of G-protein-coupled inwardly rectifying potassium current in rat periaqueductal gray neurons. *Neuroscience* 90(1): 209-19.

Hanna, C. (1960). A demonstration of morphine tolerance and physical dependence in the rat. *Arch Int Pharmacodyn Ther* 124: 326-9.

Hara, H., H. Onodera, et al. (1992). Effects of aging on signal transmission and transduction systems in the gerbil brain: morphological and autoradiographic study. *Neuroscience* 46(2): 475-88.

Hardman, J., L. Limbird, et al., Eds. (2001). Goodman & gilman's the pharmacological basis of therapeutics. New York, McGraw-Hill.

Harlan, R. E., S. R. Kailas, et al. (2004). Morphine actions in the rat forebrain: role of protein kinase C. *Brain Res Bull* 62(4): 285-95.

Hashimoto, T., K. Ase, et al. (1988). Postnatal development of a brain-specific subspecies of protein kinase C in rat. *J Neurosci* 8(5): 1678-83.

Hausken, Z. E., V. M. Coghlan, et al. (1994). Type II regulatory subunit (RII) of the cAMP-dependent protein kinase interaction with A-kinase anchor proteins requires isoleucines 3 and 5. *J Biol Chem* 269(39): 24245-51.

Hayward, M. D., R. S. Duman, et al. (1990). Induction of the c-fos proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Res* 525(2): 256-66.

Heller, W. T., D. Vigil, et al. (2004). C subunits binding to the protein kinase A RI alpha dimer induce a large conformational change. *J Biol Chem* 279(18): 19084-90.

Hepburn, M. J., P. J. Little, et al. (1997). Differential effects of naltrindole on morphine-induced tolerance and physical dependence in rats. *J Pharmacol Exp Ther* 281(3): 1350-6.

Herms, J., U. Zurmohle, et al. (1993). Ca2+/calmodulin protein kinase and protein kinase C expression during development of rat hippocampus. *Dev Neurosci* 15(6): 410-6.

Hescheler, J., W. Rosenthal, et al. (1987). The GTP-binding protein, Go, regulates neuronal calcium channels. *Nature* 325(6103): 445-7.

Hovious, J. R. and M. A. Peters (1985). Opiate self-administration in adult offspring of methadone-treated female rats. *Pharmacol Biochem Behav* 22(6): 949-53.

Ihnatovych, I., J. Novotny, et al. (2002a). Opposing changes of trimeric G protein levels during ontogenetic development of rat brain. *Brain Res Dev Brain Res* 133(1): 57-67.

Ihnatovych, I., J. Novotny, et al. (2002b). Ontogenetic development of the G protein-mediated adenylyl cyclase signalling in rat brain. *Brain Res Dev Brain Res* 133(1): 69-75.

Ikemoto, M. J., K. Inoue, et al. (2002). Identification of addicsin/GTRAP3-18 as a chronic morphine-augmented gene in amygdala. *Neuroreport* 13(16): 2079-84.

Ingram, S. L., C. W. Vaughan, et al. (1998). Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. *J Neurosci* 18(24): 10269-76.

Inoue, M. and H. Ueda (2000). Protein kinase C-mediated acute tolerance to peripheral mu-opioid analgesia in the bradykinin-nociception test in mice. *J Pharmacol Exp Ther* 293(2): 662-9.

Inturrisi, C. E. (1999). Preclinical evidence for a role of glutamatergic systems in opioid tolerance and dependence. *Seminars in Neuroscience* 9: 110-119.

Jackson, H. C. and I. Kitchen (1989). Behavioural effects of selective mu-, kappa-, and delta-opioid agonists in neonatal rats. *Psychopharmacology* 97(3): 404-9.

Javed, R. R., W. L. Dewey, et al. (2004). PKC and PKA inhibitors reverse tolerance to morphine-induced hypothermia and supraspinal analgesia in mice. *Eur J Pharmacol* 492(2-3): 149-57.

Jiang, X., M. U. Naik, et al. (1994). Developmental expression of the protein kinase C family in rat hippocampus. *Brain Res Dev Brain Res* 78(2): 291-5.

Johnstone, L. E., C. H. Brown, et al. (2000). Local morphine withdrawal increases c-fos gene, Fos protein, and oxytocin gene expression in hypothalamic magnocellular neurosecretory cells. *J Neurosci* 20(3): 1272-80.

Jones, K. L. and G. A. Barr (1995). Ontogeny of morphine withdrawal in the rat. *Behav Neurosci* 109(6): 1189-98.

Jones, K. L. and G. A. Barr (2000). Opiate withdrawal in the fetal rat: a behavioral profile. *Pharmacol Biochem Behav* 66(2): 419-24.

Jones, K. L. and G. A. Barr (2001). Injections of an opioid antagonist into the locus coeruleus and periaqueductal gray but not the amygdala precipitates morphine withdrawal in the 7-day-old rat. *Synapse* 39(2): 139-51.

Jones, K. L., H. Zhu, et al. (2002). Attenuation of acute morphine withdrawal in the neonatal rat by the competitive NMDA receptor antagonist LY235959. *Neuropsychopharmacology* 26(3): 301-10.

Joyce, M. P. and G. A. Barr (1995). Ontogeny of Fos protein-like immunoreactivity in the suprachiasmatic nucleus. *Synapse* 21(1): 54-9.

Julien, R. (1998). A primer of drug action: a concise, nontechnical guide of the actions, uses, and side effects of psychoactive drugs. New York, W. H. Feeman and Company.

Kandel, E. R., J. H. Schwartz, et al. (2000). Principles of neural science. New York, McGraw-Hill.

Katada, T., A. G. Gilman, et al. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur J Biochem* 151(2): 431-7.

Kato, H., I. Uno, et al. (1989). Activation of phosphatidylinositol kinase and phosphatidylinositol-4-phosphate kinase by cAMP in Saccharomyces cerevisiae. *J Biol Chem* 264(6): 3116-21.

Kenner, C. and K. D'Apolito (1997). Outcomes for children exposed to drugs in utero. *J Obstet Gynecol Neonatal Nurs* 26(5): 595-603.

Kim, C., N. H. Xuong, et al. (2005). Crystal structure of a complex between the catalytic and regulatory (RIalpha) subunits of PKA. *Science* 307(5710): 690-6.

Kimes, A. S. and E. D. London (1989). Glucose utilization in the rat brain during chronic morphine treatment and naloxone-precipitated morphine withdrawal. *J Pharmacol Exp Ther* 248(2): 538-45.

Koob, G. F. and F. E. Bloom (1988). Cellular and molecular mechanisms of drug dependence. *Science* 242(4879): 715-23.

Koob, G. F., R. Maldonado, et al. (1992). Neural substrates of opiate withdrawal. *Trends Neurosci* 15(5): 186-91.

Koob, G. F., T. L. Wall, et al. (1989). Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal. *Psychopharmacology* (Berl) 98(4): 530-4.

Korf, J., B. S. Bunney, et al. (1974). Noradrenergic neurons: morphine inhibition of spontaneous activity. *Eur J Pharmacol* 25(2): 165-9.

Kornblum, H. I., D. E. Hurlbut, et al. (1987). Postnatal development of multiple opioid receptors in rat brain. *Brain Res* 465(1-2): 21-41.

Kramer, H. K. and E. J. Simon (1999b). Role of protein kinase C (PKC) in agonist-induced mu-opioid receptor down-regulation: I. PKC translocation to the membrane of SH-SY5Y neuroblastoma cells is induced by mu-opioid agonists. *J Neurochem* 72(2): 585-93.

Kreek, M. J. (1996). Cocaine, dopamine and the endogenous opioid system. *J Addict Dis* 15(4): 73-96.

Kuwahara, M. D. and S. B. Sparber (1981). Prenatal withdrawal from opiates interferes with hatching of otherwise viable chick fetuses. *Science* 212(4497): 945-7.

Lane-Ladd, S. B., J. Pineda, et al. (1997). CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J Neurosci* 17(20): 7890-901.

Laschka, E. and A. Herz (1977). Sites of action of morphine involved in the development of physical dependence in rats. III. Autoradiographic studies. *Psychopharmacology* (Berl) 53(1): 33-7.

Laschka, E., H. Teschemacher, et al. (1976b). Sites of action of morphine involved in the development of physical dependence in rats. II. Morphine withdrawal precipitated by application of morphine antagonists into restricted parts of the ventricular system and by microinjection into various brain areas. *Psychopharmacologia* 46(2): 141-7.

Law, P. Y., D. S. Hom, et al. (1983). Opiate receptor down-regulation and desensitization in neuroblastoma X glioma NG108-15 hybrid cells are two separate cellular adaptation processes. *Mol Pharmacol* 24(3): 413-24.

Le Guen, S., C. Gestreau, et al. (2001). Sensitivity to naloxone of the behavioral signs of morphine withdrawal and c-Fos expression in the rat CNS: a quantitative dose-response analysis. *J Comp Neurol* 433(2): 272-96.

Le Guen, S., C. Gestreau, et al. (2003). Morphine withdrawal precipitated by specific mu, delta or kappa opioid receptor antagonists: a c-Fos protein study in the rat central nervous system. *Eur J Neurosci* 17(11): 2425-37.

Lee, E. J., H. Kato, et al. (1994). Ontogeny of protein kinase C in the rat hippocampus: an autoradiographic study with [3H]phorbol 12,13-dibutyrate. *Brain Res* 665(1): 127-9.

Lee, J. J., E. T. Hahm, et al. (2004). Activation of protein kinase C antagonizes the opioid inhibition of calcium current in rat spinal dorsal horn neurons. *Brain Res* 1017(1-2): 108-19.

Leshner, A. I. (1996). Understanding drug addiction: implications for treatment. *Hosp Pract* (Off Ed) 31(10): 47-54, 57-9.

Levine, R. R. (2000). Pharmacology: drug actions and reactions. New York, The Parthenon Publishing Group.

Levinthal, C. (1996). Drugs, behavior, and modern society. Boston, Allyn and Bacon.

Li, Y. and S. C. Roerig (1999). Alteration of spinal protein kinase C expression and kinetics in morphine, but not clonidine, tolerance. *Biochem Pharmacol* 58(3): 493-501.

Lichtblau, L. and S. B. Sparber (1981). Opiate withdrawal in utero increases neonatal morbidity in the rat. *Science* 212(4497): 943-5.

Liu, J. G. and K. J. Anand (2001). Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. *Brain Res Brain Res Rev* 38(1-2): 1-19.

Liu, Q. R., P. W. Zhang, et al. (2002). KEPI, a PKC-dependent protein phosphatase 1 inhibitor regulated by morphine. *J Biol Chem* 277(15): 13312-20.

Lord, J. A., A. A. Waterfield, et al. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature* 267(5611): 495-9.

Lotti, V. J., P. Lomax, et al. (1966). Acute tolerance to morphine following systemic and intracerebral injection in the rat. *Int J Neuropharmacol* 5(1): 35-42.

Lou, L., T. Zhou, et al. (1999). Modulation of Ca2+/calmodulin-dependent protein kinase II activity by acute and chronic morphine administration in rat hippocampus: differential regulation of alpha and beta isoforms. *Mol Pharmacol* 55(3): 557-63.

Maeda, T., S. Kishioka, et al. (2002). Naloxone-Precipitated Morphine Withdrawal Elicits Increases in c-fos mRNA Expression in Restricted Regions of the Infant Rat Brain. *Jpn J Pharmacol* 90(3): 270-5.

Makimura, M., M. Narita, et al. (1995). Influence of chronic opioid treatment on low Km GTPase activity in rat brain: evidence for the involvement of G-proteins in opioid tolerance. *Brain Res Bull* 38(6): 565-8.

Maldonado, R., J. A. Blendy, et al. (1996). Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 273(5275): 657-9.

Maldonado, R., J. Feger, et al. (1990). Differences in physical dependence induced by selective mu or delta opioid agonists and by endogenous enkephalins protected by peptidase inhibitors. *Brain Res* 520(1-2): 247-54.

Maldonado, R., M. C. Fournie-Zaluski, et al. (1992a). Attenuation of the morphine withdrawal syndrome by inhibition of catabolism of endogenous enkephalins in the periaqueductal gray matter. *Naunyn Schmiedebergs Arch Pharmacol* 345(4): 466-72.

Maldonado, R. and G. F. Koob (1993). Destruction of the locus coeruleus decreases physical signs of opiate withdrawal. *Brain Res* 605(1): 128-38.

Maldonado, R., S. Negus, et al. (1992b). Precipitation of morphine withdrawal syndrome in rats by administration of mu-, delta- and kappa-selective opioid antagonists. *Neuropharmacology* 31(12): 1231-41.

Maldonado, R., L. Stinus, et al. (1992c). Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *J Pharmacol Exp Ther* 261(2): 669-77.

Maldonado, R., O. Valverde, et al. (1995). Protein kinases in the locus coeruleus and periaqueductal gray matter are involved in the expression of opiate withdrawal. *Naunyn Schmiedebergs Arch Pharmacol* 352(5): 565-75.

Mangoura, D. and G. Dawson (1993). Opioid peptides activate phospholipase D and protein kinase C-epsilon in chicken embryo neuron cultures. *Proc Natl Acad Sci U S A* 90(7): 2915-9.

Mannelli, P., E. Gottheil, et al. (2004). Chronic very low dose naltrexone administration attenuates opioid withdrawal expression. *Biol Psychiatry* 56(4): 261-8.

Manning, B. H., N. M. Merin, et al. (2001). Reduction in opioid- and cannabinoid-induced antinociception in rhesus monkeys after bilateral lesions of the amygdaloid complex. *J Neurosci* 21(20): 8238-46.

Mansour, A., C. A. Fox, et al. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci* 18(1): 22-9.

Mao, J., D. D. Price, et al. (1995). Increases in protein kinase C gamma immunoreactivity in the spinal cord dorsal horn of rats with painful mononeuropathy. *Neurosci Lett* 198(2): 75-8.

Marcus, J., S. L. Hans, et al. (1984). A longitudinal study of offspring born to methadone-maintained women. III. Effects of multiple risk factors on development at 4, 8, and 12 months. *Am J Drug Alcohol Abuse* 10(2): 195-207.

Margolis, E. B., G. O. Hjelmstad, et al. (2003). Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. *J Neurosci* 23(31): 9981-6.

Martin, G., S. H. Ahmed, et al. (1999a). Chronic morphine treatment alters NMDA receptor-mediated synaptic transmission in the nucleus accumbens. *J Neurosci* 19(20): 9081-9.

Martin, W. R., C. G. Eades, et al. (1976). The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* 197(3): 517-32.

Martin, W. R., A. Wikler, et al. (1963). Tolerance to and Physical Dependence on Morphine in Rats. *Psychopharmacologia* 65: 247-60.

Matthes, H. W., R. Maldonado, et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383(6603): 819-23.

Mayer, D. J., J. Mao, et al. (1995). The development of morphine tolerance and dependence is associated with translocation of protein kinase C. *Pain* 61(3): 365-74.

McClung, C. A., E. J. Nestler, et al. (2005). Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *J Neurosci* 25(25): 6005-15.

McDowell, J. and I. Kitchen (1987). Development of opioid systems: peptides, receptors and pharmacology. *Brain Res* 434(4): 397-421.

McNally, G. P., M. Pigg, et al. (2004). Opioid receptors in the midbrain periaqueductal gray regulate extinction of pavlovian fear conditioning. *J Neurosci* 24(31): 6912-6919.

McPhie, A. A. and G. A. Barr (2000). The role of opioid receptors in morphine withdrawal in the infant rat. *Brain Res Dev Brain Res* 124(1-2): 73-80.

McPhie, A. A. and G. A. Barr (2003). Regional expression of the cellular protein fos induced by morphine withrawal in the 7-day old rat. *Brain Res Dev Brain Res* (Submitted manuscript).

Mestek, A., J. H. Hurley, et al. (1995). The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. *J Neurosci* 15(3 Pt 2): 2396-406.

Miyamae, T., N. Fukushima, et al. (1993). Delta opioid receptor mediates phospholipase C activation via Gi in Xenopus oocytes. *FEBS Lett* 333(3): 311-4.

Moncada, A., C. M. Cendan, et al. (2003). Effects of serine/threonine protein phosphatase inhibitors on morphine-induced antinociception in the tail flick test in mice. *Eur J Pharmaco*l 465(1-2): 53-60.

Morgan, J. I., D. R. Cohen, et al. (1987). Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* 237(4811): 192-7.

Muller, D. L. and E. M. Unterwald (2004). In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther* 310(2): 774-82.

Muranyi, A., P. Gergely, et al. (1997). The possible role of protein phosphatase 2A in the sodium sensitivity of the receptor binding of opiate antagonists naloxone and naltrindole. *Brain Res Bull* 44(3): 273-9.

Narita, M., Y. Feng, et al. (1994a). A protein kinase inhibitor, H-7, inhibits the development of tolerance to opioid antinociception. *Eur J Pharmacol* 271(2-3): 543-5.

Narita, M., Y. Feng, et al. (1994b). Repeated administration of opioids alters characteristics of membrane-bound phorbol ester binding in rat brain. *Eur J Pharmacol* 271(2-3): 547-50.

Narita, M., M. Makimura, et al. (1994c). Influence of chronic morphine treatment on protein kinase C activity: comparison with butorphanol and implication for opioid tolerance. *Brain Res* 650(1): 175-9.

Narita, M., H. Mizoguchi, et al. (1996). Role of protein kinase C in desensitization of spinal delta-opioid-mediated antinociception in the mouse. *Br J Pharmacol* 118(7): 1829-35.

Narita, M., H. Mizoguchi, et al. (2001a). Involvement of spinal protein kinase Cgamma in the attenuation of opioid mu-receptor-mediated G-protein activation after chronic intrathecal administration of [D-Ala2,N-MePhe4,Gly-Ol(5)]enkephalin. *J Neurosci* 21(11): 3715-20.

Narita, M., H. Mizoguchi, et al. (1995). Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered mu-opioid receptor agonist in the mouse. *Eur J Pharmacol* 280(2): R1-3.

Narita, M., K. Mizuo, et al. (2002). Up-regulation of the G(q/11alpha) protein and protein kinase C during the development of sensitization to morphine-induced hyperlocomotion. *Neuroscience* 111(1): 127-32.

Narita, M., M. Suzuki, et al. (2004b). Neuronal protein kinase C gamma-dependent proliferation and hypertrophy of spinal cord astrocytes following repeated in vivo administration of morphine. *Eur J Neurosci* 19(2): 479-84.

Nestler, E. J. (1992). Molecular mechanisms of drug addiction. *J Neurosci* 12(7): 2439-50.

Nestler, E. J. (2001). Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* 2(2): 119-28.

Nestler, E. J. (2004). Molecular mechanisms of drug addiction. *Neuropharmacology 47 Suppl* 1: 24-32.

Nestler, E. J. and G. K. Aghajanian (1997). Molecular and cellular basis of addiction. *Science* 278(5335): 58-63.

Nestler, E. J., M. Alreja, et al. (1994). Molecular and cellular mechanisms of opiate action: studies in the rat locus coeruleus. *Brain Res Bull* 35(5-6): 521-8.

Nestler, E. J., J. J. Erdos, et al. (1989). Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain Res* 476(2): 230-9.

Nestler, E. J. and J. F. Tallman (1988). Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. *Mol Pharmacol* 33(2): 127-32.

North, R. A., J. T. Williams, et al. (1987). Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci U S A* 84(15): 5487-91.

Nye, H. E. and E. J. Nestler (1996). Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. *Mol Pharmacol* 49(4): 636-45.

Office of Applied Studies, S. (2001). The DASIS report: heroin-changes in how it is used.

Oommen, A. and D. J. A. Anand (1995). The rat brain delta opioid receptor (ontogenic studies). *Analgesia* 1: 619-622.

Ornoy, A., V. Michailevskaya, et al. (1996). The developmental outcome of children born to heroin-dependent mothers, raised at home or adopted. *Child Abuse Negl* 20(5): 385-96.

Parolaro, D., T. Rubino, et al. (1993). In situ hybridization reveals specific increases in G alpha s and G alpha o mRNA in discrete brain regions of morphine-tolerant rats. *Eur J Pharmacol* 244(3): 211-22.

Pasternak, G. W., Y. A. Kolesnikov, et al. (1995). Perspectives on the N-methyl-D-aspartate/nitric oxide cascade and opioid tolerance. *Neuropsychopharmacology* 13(4): 309-13.

Penn, R. B., A. N. Pronin, et al. (2000). Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med* 10(2): 81-9.

Pennypacker, K. R., P. M. Hudson, et al. (1995). DNA binding activity of CREB transcription factors during ontogeny of the central nervous system. *Brain Res Dev Brain Res* 86(1-2): 242-9.

Pert, C. B. and S. H. Snyder (1973). Opiate receptor: demonstration in nervous tissue. *Science* 179(77): 1011-4.

Peters, M. A. (1975). Development of a blood-brain barrier to methadone in the newborn rat. *J Pharmacol Exp Ther* 192(3): 513-20.

Policy, O. o. N. D. C. (2001). The economic costs of drug abuse in the united states, 1992-1998. Washington, DC, The Executive Office of the President.

Pothos, E., P. Rada, et al. (1991). Dopamine microdialysis in the nucleus accumbens during acute and chronic morphine, naloxone-precipitated withdrawal and clonidine treatment. *Brain Res* 566(1-2): 348-50.

Przewlocka, B., J. Turchan, et al. (1996). The effect of single and repeated morphine administration on the prodynorphin system activity in the nucleus accumbens and striatum of the rat. *Neuroscience* 70(3): 749-54.

Punch, L. J., D. W. Self, et al. (1997). Opposite modulation of opiate withdrawal behaviors on microinfusion of a protein kinase A inhibitor versus activator into the locus coeruleus or periaqueductal gray. *J Neurosci* 17(21): 8520-7.

Rajegowda, B. K., L. Glass, et al. (1972). Methadone withdrawal in newborn infants. *J Pediatr* 81(3): 532-4.

Rasmussen, K., D. B. Beitner-Johnson, et al. (1990). Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates. *J Neurosci* 10(7): 2308-17.

Redmond, D. E., Jr. and J. H. Krystal (1984). Multiple mechanisms of withdrawal from opioid drugs. *Annu Rev Neurosci* 7: 443-78.

Reed, M. D. and J. B. Besunder (1989). Developmental pharmacology: ontogenic basis of drug disposition. *Pediatr Clin North Am* 36(5): 1053-74.

Rimanoczy, A., R. Slamberova, et al. (2001). Prenatal morphine exposure alters estrogen regulation of kappa receptors in the cortex and POA of adult female rats but has no effects on these receptors in adult male rats. *Brain Res* 894(1): 154-6.

Rimanoczy, A. and I. Vathy (1995). Prenatal exposure to morphine alters brain mu opioid receptor characteristics in rats. *Brain Res* 690(2): 245-8.

Rius, R. A., J. Barg, et al. (1991a). The prenatal development profile of expression of opioid peptides and receptors in the mouse brain. *Brain Res Dev Brain Res* 58(2): 237-41.

Rius, R. A., S. Mollner, et al. (1994). Developmental changes in Gs and G(olf) proteins and adenylyl cyclases in mouse brain membranes. *Brain Res* 643(1-2): 50-8.

Rius, R. A., R. A. Streaty, et al. (1991b). Developmental expression of G proteins that differentially modulate adenylyl cyclase activity in mouse brain. *FEBS Lett* 288(1-2): 51-4.

Rodriguez Parkitna, J. M., W. Bilecki, et al. (2004). Effects of morphine on gene expression in the rat amygdala. *J Neurochem* 91(1): 38-48.

Rohde, D. S., D. J. Detweiler, et al. (1996). Spinal cord mechanisms of opioid tolerance and dependence: Fos-like immunoreactivity increases in subpopulations of spinal cord neurons during withdrawal [corrected]. *Neuroscience* 72(1): 233-42.

Rosen, T. S. and H. L. Johnson (1982). Children of methadone-maintained mothers: follow-up to 18 months of age. *J Pediatr* 101(2): 192-6.

Rosenfeld, G. C. and T. F. Burks (1977). Single-dose tolerance to morphine hypothermia in the rat: differentiation of acute from long-term tolerance. *J Pharmacol Exp Ther* 202(3): 654-9.

Rossetti, Z. L., Y. Hmaidan, et al. (1992). Marked inhibition of mesolimbic dopamine release: a common feature of ethanol, morphine, cocaine and amphetamine abstinence in rats. *Eur J Pharmacol* 221(2-3): 227-34.

Rothman, R. B., J. A. Danks, et al. (1986). Morphine tolerance increases mu-noncompetitive delta binding sites. *Eur J Pharmacol* 124(1-2): 113-9.

Rothstein, P. and J. B. Gould (1974). Born with a habit. Infants of drug-addicted mothers. *Pediatr Clin North Am* 21(2): 307-21.

Rubini, P., R. Schulz, et al. (1982). Opiate receptor binding studies in the mouse vas deferens exhibiting tolerance without dependence. *Naunyn Schmiedebergs Arch Pharmacol* 319(2): 142-6.

Salem, A. and W. Hope (1999). Role of endogenous adenosine in the expression of opiate withdrawal in rats. *Eur J Pharmacol* 369(1): 39-42.

Sanchez-Blazquez, P., M. D. Rodriguez, et al. (1996). Antibodies directed against the mu-opioid receptor alleviated multiple signs of morphine withdrawal in mice. *Life Sci* 59(8): PL87-92.

Satoh, M. and M. Minami (1995). Molecular pharmacology of the opioid receptors. *Pharmacol Ther* 68(3): 343-64.

Schulteis, G., C. J. Heyser, et al. (1997). Opiate withdrawal signs precipitated by naloxone following a single exposure to morphine: potentiation with a second morphine exposure. *Psychopharmacology* (Berl) 129(1): 56-65.

Schulz, R., E. Seidl, et al. (1982). Opioid dependence and cross-dependence in the isolated guinea-pig ileum. *Eur J Pharmacol* 84(1-2): 33-40.

Schulz, R., M. Wuster, et al. (1980). Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. *Nature* 285(5762): 242-3.

Shahak, H., T. A. Slotkin, et al. (2003). Alterations in PKCgamma in the mouse hippocampus after prenatal exposure to heroin: a link from cell signaling to behavioral outcome. *Brain Res Dev Brain Res* 140(1): 117-25.

Sharma, S. K., W. A. Klee, et al. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* 72(8): 3092-6.

Sharma, S. K., W. A. Klee, et al. (1977). Opiate-dependent modulation of adenylate cyclase. *Proc Natl Acad Sci U S A* 74(8): 3365-9.

Shaw-Lutchman, T. Z., M. Barrot, et al. (2002). Regional and cellular mapping of cAMP response element-mediated transcription during naltrexone-precipitated morphine withdrawal. *J Neurosci* 22(9): 3663-72.

Sheng, M. and M. E. Greenberg (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* 4(4): 477-85.

Shippenberg, T. S. and G. I. Elmer (1998). The neurobiology of opiate reinforcement. *Crit Rev Neurobiol* 12(4): 267-303.

Siddiqui, A., S. Haq, et al. (1997). Perinatal exposure to morphine disrupts brain norepinephrine, ovarian cyclicity, and sexual receptivity in rats. *Pharmacol Biochem Behav* 58(1): 243-8.

Sim-Selley, L. J., D. E. Selley, et al. (2000). Chronic heroin self-administration desensitizes mu opioid receptor-activated G-proteins in specific regions of rat brain. *J Neurosci* 20(12): 4555-62.

Simon, E. J., J. M. Hiller, et al. (1973). Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc Natl Acad Sci U S A* 70(7): 1947-9.

Simonin, F., O. Valverde, et al. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *Embo J* 17(4): 886-97.

Slotkin, T. A., F. J. Seidler, et al. (2001). Heroin neuroteratogenicity: targeting adenylyl cyclase as an underlying biochemical mechanism. *Brain Res Dev Brain Res* 132(1): 69-79.

Smart, D. and D. G. Lambert (1996a). delta-Opioids stimulate inositol 1,4,5-trisphosphate formation, and so mobilize Ca2+ from intracellular stores, in undifferentiated NG108-15 cells. *J Neurochem* 66(4): 1462-7.

Smith, F. L., D. S. Dombrowski, et al. (1999a). Involvement of intracellular calcium in morphine tolerance in mice. *Pharmacol Biochem Behav* 62(2): 381-8.

Smith, F. L., R. Javed, et al. (2002). Prolonged reversal of morphine tolerance with no reversal of dependence by protein kinase C inhibitors. *Brain Res* 958(1): 28-35.

Smith, F. L., R. R. Javed, et al. (2003). The expression of a high level of morphine antinociceptive tolerance in mice involves both PKC and PKA. *Brain Res* 985(1): 78-88.

Smith, F. L., A. B. Lohmann, et al. (1999b). Involvement of phospholipid signal transduction pathways in morphine tolerance in mice. *Br J Pharmacol* 128(1): 220-6.

Smith, F. L., P. A. Smith, et al. (2004). Effects of mGlu1 and mGlu5 metabotropic glutamate antagonists to reverse morphine tolerance in mice. *Eur J Pharmacol* 492(2-3): 137-42.

Soepatmi, S. (1994). Developmental outcomes of children of mothers dependent on heroin or heroin/methadone during pregnancy. *Acta Paediatr Suppl* 404: 36-9.

Spanagel, R., O. F. Almeida, et al. (1994). Endogenous kappa-opioid systems in opiate withdrawal: role in aversion and accompanying changes in mesolimbic dopamine release. *Psychopharmacology* (Berl) 115(1-2): 121-7.

Sparber, S. B. and L. Lichtblau (1983). Postnatal abstinence or acute toxicity can account for morbidity in developmental studies with opiates. *Life Sci* 33(12): 1135-40.

Stefano, G. B., Y. Goumon, et al. (2000). Endogenous morphine. *Trends Neurosci* 23(9): 436-42.

Steingart, R. A., J. Barg, et al. (1998). Pre- and postsynaptic alterations in the septohippocampal cholinergic innervations after prenatal exposure to drugs. *Brain Res Bull* 46(3): 203-9.

Steingart, R. A., W. F. Silverman, et al. (2000b). Neural grafting reverses prenatal drug-induced alterations in hippocampal PKC and related behavioral deficits. *Brain Res Dev Brain Res* 125(1-2): 9-19.

Sternini, C., M. Spann, et al. (1996). Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc Natl Acad Sci U S A* 93(17): 9241-6.

Stevens, C. W. (1994). Perspectives on opioid tolerance from basic research: behavioural studies after spinal administration in rodents. *Cancer Surv* 21: 25-47.

Stevens, C. W. and T. L. Yaksh (1989). Magnitude of opioid dependence after continuous intrathecal infusion of mu- and delta-selective opioids in the rat. *Eur J Pharmacol* 166(3): 467-72.

Stinus, L., M. Le Moal, et al. (1990). Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. *Neuroscience* 37(3): 767-73.

Stornetta, R. L., F. E. Norton, et al. (1993). Autonomic areas of rat brain exhibit increased Fos-like immunoreactivity during opiate withdrawal in rats. *Brain Res* 624(1-2): 19-28.

Suresh, S. and K. J. Anand (1998). Opioid tolerance in neonates: mechanisms, diagnosis, assessment, and management. *Semin Perinatol* 22(5): 425-33.

Suzuki, T., M. Narita, et al. (1990). Interaction between mu and kappa receptors in pharmacological effects of tolerance to and dependence on several opioids. *J Pharmacobio-Dyn* 13: s133.

Suzuki, T., M. Narita, et al. (1992). Effects of nor-binaltorphimine on the development of analgesic tolerance to and physical dependence on morphine. *Eur J Pharmacol* 213(1): 91-7.

Szeto, H. H. (1995). Opioid receptor approaches for the development of medications for pregnant women. *NIDA Res Monogr* 149: 100-15.

Szücs, M. and C. J. Coscia (1990). Evidence for delta-opioid binding and GTP-regulatory proteins in 5-day-old rat brain membranes. *J Neurochem* 54(4): 1419-25.

Tan, M., M. Groszer, et al. (2003). Phosphoinositide 3-Kinase Cascade Facilitates {micro}-Opioid Desensitization in Sensory Neurons by Altering G-Protein-Effector Interactions. *J Neurosci* 23(32): 10292-10301.

Tasken, K. and E. M. Aandahl (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev* 84(1): 137-67.

Tempel, A., J. Habas, et al. (1988). Morphine-induced downregulation of mu-opioid receptors in neonatal rat brain. *Brain Res* 469(1-2): 129-33.

Tempel, A. and R. S. Zukin (1987). Neuroanatomical patterns of the mu, delta, and kappa opioid receptors of rat brain as determined by quantitative in vitro autoradiography. *Proc Natl Acad Sci U S A* 84(12): 4308-12.

Terwilliger, R. Z., D. Beitner-Johnson, et al. (1991). A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 548(1-2): 100-10.

Thornton, S. R., A. F. Wang, et al. (1997). Characterization of neonatal rat morphine tolerance and dependence. *Eur J Pharmacol* 340(2-3): 161-7.

Tobias, J. D., C. L. Schleien, et al. (1990). Methadone as treatment for iatrogenic narcotic dependency in pediatric intensive care unit patients. *Crit Care Med* 18(11): 1292-3.

Tokuyama, S., Y. Feng, et al. (1995a). Possible involvement of protein kinases in physical dependence on opioids: studies using protein kinase inhibitors, H-7 and H-8. *Eur J Pharmacol* 284(1-2): 101-7.

Tokuyama, S., I. K. Ho, et al. (2000). A protein kinase inhibitor, H-7, blocks naloxone-precipitated changes in dopamine and its metabolites in the brains of opioid-dependent rats. *Brain Res Bull* 52(5): 363-9.

Tortella, F. C., J. E. Moreton, et al. (1979). Electroencephalographic and behavioral tolerance to and cross-tolerance between D-Ala2-methionine-enkephalinamide and morphine in the rat. *J Pharmacol Exp Ther* 210(2): 174-9.

Tortorici, V., C. S. Robbins, et al. (1999). Tolerance to the antinociceptive effect of morphine microinjections into the ventral but not lateral-dorsal periaqueductal gray of the rat. *Behav Neurosci* 113(4): 833-9.

Traber, J., R. Gullis, et al. (1975). Influence of opiates on the levels of adenosine 3':5'-cyclic monophosphate in neuroblastoma X glioma hybrid cells. Life Sci 16(12): 1863-8.

Trujillo, K. A. (1995). Effects of noncompetitive N-methyl-D-aspartate receptor antagonists on opiate tolerance and physical dependence. *Neuropsychopharmacology* 13(4): 301-7.

Trujillo, K. A. and H. Akil (1991). Opiate tolerance and dependence: recent findings and synthesis. *New Biol* 3(10): 915-23.

Turner, R. S., R. L. Raynor, et al. (1984). Developmental studies of phospholipid-sensitive Ca2+-dependent protein kinase and its substrates and of phosphoprotein phosphatases in rat brain. *Proc Natl Acad Sci U S A* 81(10): 3143-7.

Ueda, H., T. Miyamae, et al. (1995). Opioid mu- and kappa-receptor mediate phospholipase C activation through Gi1 in Xenopus oocytes. *Brain Res Mol Brain Res* 32(1): 166-70.

Van Bockstaele, E. J., E. E. Colago, et al. (1996). Ultrastructural evidence for prominent distribution of the mu-opioid receptor at extrasynaptic sites on noradrenergic dendrites in the rat nucleus locus coeruleus. *J Neurosci* 16(16): 5037-48.

Van Praag, H. and H. Frenk (1991). Evidence for opiate tolerance in newborn rats. *Brain Res Dev Brain Res* 60(1): 99-102.

Vathy, I., A. Rimanoczy, et al. (2000). Prenatal exposure to morphine differentially alters gonadal hormone regulation of delta-opioid receptor binding in male and female rats. *Brain Res Bull* 53(6): 793-800.

Vaughan, C. W. and M. J. Christie (1997). Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey in vitro. *J Physiol* 498(Pt 2): 463-72.

Veinante, P., M. E. Stoeckel, et al. (2003). c-Fos and peptide immunoreactivities in the central extended amygdala of morphine-dependent rats after naloxone-precipitated withdrawal. *Eur J Neurosci* 18(5): 1295-305.

Ventayol, P., X. Busquets, et al. (1997). Modulation of immunoreactive protein kinase C-alpha and beta isoforms and G proteins by acute and chronic treatments with morphine and other opiate drugs in rat brain. *Naunyn Schmiedebergs Arch Pharmacol* 355(4): 491-500.

Walters, C. L., G. Aston-Jones, et al. (2000). Expression of fos-related antigens in the nucleus accumbens during opiate withdrawal and their attenuation by a D2 dopamine receptor agonist. *Neuropsychopharmacology* 23(3): 307-15.

Walters, C. L. and J. A. Blendy (2001). Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. *J. Neurosci* 21(23): 9438-44.

Wang, Z. and W. Sadee (2000). Tolerance to morphine at the mu-opioid receptor differentially induced by cAMP-dependent protein kinase activation and morphine. *Eur J Pharmacol* 389(2-3): 165-71.

Ward, R. M. (1989). Maternal-placental-fetal unit: unique problems of pharmacologic study. *Pediatr Clin North Am* 36(5): 1075-88.

Werling, L. L., P. N. McMahon, et al. (1989). Selective changes in mu opioid receptor properties induced by chronic morphine exposure. *Proc Natl Acad Sci U S A* 86(16): 6393-7.

Widnell, K. L., D. W. Self, et al. (1996). Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* 276(1): 306-15.

Wiedenmayer, C. P. and G. A. Barr (2001). Developmental changes in c-fos expression to an age-specific social stressor in infant rats. *Behav Brain Res* 126(1-2): 147-57.

Williams, S., G. Evan, et al. (1990). Spinal c-fos induction by sensory stimulation in neonatal rats. *Neurosci Lett* 109(3): 309-14.

Wilson, D. A. and M. Leon (1988). Noradrenergic modulation of olfactory bulb excitability in the postnatal rat. *Brain Res* 470(1): 69-75.

Windh, R. T. and C. M. Kuhn (1995a). Increased sensitivity to mu opiate antinociception in the neonatal rat despite weaker receptor-guanyl nucleotide binding protein coupling. *J Pharmacol Exp Ther* 273(3): 1353-60.

Windh, R. T., P. J. Little, et al. (1995b). The ontogeny of mu opiate tolerance and dependence in the rat: antinociceptive and biochemical studies. *J Pharmacol Exp Ther* 273(3): 1361-74.

Wise, R. A. (1987). The role of reward pathways in the development of drug dependence. *Pharmacol Ther* 35(1-2): 227-63.

Wongchanapai, W., B. K. Tsang, et al. (1998). Relative involvement of spinal opioid receptors in physical dependence on intrathecal butorphanol and morphine. *Pharmacol Biochem Behav* 60(4): 899-907.

Wooten, G. F., P. DiStefano, et al. (1982). Regional cerebral glucose utilization during morphine withdrawal in the rat. *Proc Natl Acad Sci U S A* 79(10): 3360-4.

Wüster, M., R. Schulz, et al. (1985). Opioid tolerance and dependence: re-evaluating the unitary hypothesis. *Trends Pharmacol Sci*: 64-67.

Yanai, J., R. Huleihel, et al. (2003). Functional changes after prenatal opiate exposure related to opiate receptors' regulated alterations in cholinergic innervation. *Int J Neuropsychopharmacol* 6(3): 253-65.

Yanai, J., R. A. Steingart, et al. (2000). The relationship between neural alterations and behavioral deficits after prenatal exposure to heroin. *Ann N Y Acad Sci* 914: 402-11.

Yaniv, S. P., Z. Naor, et al. (2004). Prenatal heroin exposure alters cholinergic receptor stimulated activation of the PKCbetaII and PKCgamma isoforms. *Brain Res Bull* 63(4): 339-49.

Yi, D. K. and G. A. Barr (1995). The induction of Fos-like immunoreactivity by noxious thermal, mechanical and chemical stimuli in the lumbar spinal cord of infant rats. *Pain* 60(3): 257-65.

Yi, D. K. and G. A. Barr (1997). Formalin-induced c-fos expression in the spinal cord of fetal rats. *Pain* 73(3): 347-54.

Yoburn, B. C., B. Billings, et al. (1993). Opioid receptor regulation in mice. *J Pharmacol Exp Ther* 265(1): 314-20.

Yoburn, B. C., B. A. Gomes, et al. (2003). Role of G(i)alpha2-protein in opioid tolerance and mu-opioid receptor downregulation in vivo. *Synapse* 47(2): 109-16.

Yoshimasa, T., D. R. Sibley, et al. (1987). Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. *Nature* 327(6117): 67-70.

Young, G. A. and N. Khazan (1985). Comparison of abstinence syndromes following chronic administration of mu and kappa opioid agonists in the rat. *Pharmacol Biochem Behav* 23(3): 457-60.

Yu, W., J. X. Hao, et al. (1997). The development of morphine tolerance and dependence in rats with chronic pain. *Brain Res* 756(1-2): 141-6.

Zadina, J. E., A. J. Kastin, et al. (1985). Developmental, behavioral, and opiate receptor changes after prenatal or postnatal beta-endorphin, CRF, or Tyr-MIF-1. *Psychoneuroendocrinology* 10(4): 367-83.

Zamir, I. and J. Yanai (1995). GTPase activity in mouse hippocampus membranes following prenatal exposure to heroin and phenobarbital. *Biochem Pharmacol* 50(1): 127-30.

Zeitz, K. P., A. B. Malmberg, et al. (2001). Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. *Pain* 94(3): 245-53.

Zelson, C., E. Rubio, et al. (1971). Neonatal narcotic addiction: 10 year observation. *Pediatrics* 48(2): 178-89.

Zhou, X. M., P. Curran, et al. (1994). Modulation of adenylylcyclase by protein kinase C in human neurotumor SK-N-MC cells: evidence that the alpha isozyme mediates both potentiation and desensitization. *J Neurochem* 63(4): 1361-70.

Zhu, H. (2002). Ontogeny of glutamate receptors mediated morphine tolerance and withdrawal in rats. Psychology Department. New York, The City University of New York: 63-81.

Zhu, H. and G. A. Barr (2000). Naltrexone-precipitated morphine withdrawal in infant rat is attenuated by acute administration of NOS inhibitors but not NMDA receptor antagonists. *Psychopharmacology* (Berl) 150(3): 325-36.

Zhu, H. and G. A. Barr (2001a). Inhibition of morphine withdrawal by the NMDA receptor antagonist MK- 801 in rat is age-dependent. *Synapse* 40(4): 282-93.

Zhu, H. and G. A. Barr (2003a). Ontogeny of NMDA receptor-mediated morphine tolerance in the postnatal rat. *Pain* 104(3): 437-47.

Zhu, H., S. Jenab, et al. (2003b). The clinically available NMDA receptor antagonist dextromethorphan attenuates acute morphine withdrawal in the neonatal rat. *Brain Res Dev Brain Res* 142(2): 209-13.

Zhu, H. and W. Zhou (2001c). Morphine induces synchronous oscillatory discharges in the rat locus coeruleus. *J Neurosci* 21(21): RC179.

Zhu, Y., M. S. Hsu, et al. (1998a). Developmental expression of the mu, kappa, and delta opioid receptor mRNAs in mouse. *J Neurosci* 18(7): 2538-49.