INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

THE DEVELOPMENT OF MORPHINE WITHDRAWAL IN THE FETAL AND INFANT RAT

by Kathy L. Jones

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.

1997

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.

April 30, 1997
Date

Gordon ABARR
Chair of Examining Committee

April 30, 1997
Date

Executive Officer

Dr. Victoria Luine

Dr. Gerald Turkewitz

Dr. Eliot Gardner

Dr. Cynthia Kuhn
Supervisory Committee

The City University of New York

Abstract

THE DEVELOPMENT OF MORPHINE WITHDRAWAL IN THE FETAL AND NEONATAL RAT

By

Kathy L. Jones

Advisor: Professor Gordon A. Barr

Of the neurobehavioral effects that can be produced by opiate drugs, some of the most serious by far are the complex set of symptoms that comprise the neonatal abstinence syndrome. The experiments of this thesis were therefore designed to construct a model to allow further investigation of the opiate withdrawal syndrome in the developing animal. The hypothesis of this thesis is that both the fetus and infant are capable of experiencing opiate withdrawal when the age specific behavioral repertoire to the age of the animal is examined. The goal was therefore to describe in detail the unconditioned behavioral changes that follow precipitated withdrawal from chronic exposure to morphine during development, including the fetus, the neonate, and continuing through adolescence and to define specific anatomical mechanisms that may mediate the withdrawal syndrome in the rat.

This was a 2-stage process. In the first stage, the dam or the rat pup was treated chronically with morphine. Controls were saline treated animals. Injections of naltrexone were given to the fetus or pup to precipitate withdrawal. Behavioral changes were then assessed from fetal age through adolescence. Postnatal animals were tested at age 7, 14, 21, and 42. Fetuses were tested *in utero* at gestation day 20. Three major developmental patterns of withdrawal emerged. There were abstinence behaviors that were unique to the 7 or 14 day old pup. These included head swaying, hindpaw movements, rolling, and stretching. Other behaviors appeared only in the 21 or 42 day old pup. These behaviors were burrowing, diarrhea, jumping, teeth chattering, and wet dog shakes and constitute the adult withdrawal syndrome. Finally, there were behaviors labeled developmentally continuous behaviors that were characteristic of animals in withdrawal at all ages. These included walking forward and failure to be quiet. Morphine exposed fetuses that were treated with naltrexone showed an increase in behaviors such as body curls, face wiping, forelimb movements, mouth movements, and were quiet less often as compared to control animals. These results indicate that morphine abstinent rats demonstrate withdrawal behaviors that are within the developmental repertoire of the pup.

The goal of the second stage was to examine the role of specific neural

sites in the precipitated opiate withdrawal syndrome in the 7 day old rat, concentrating on three brain regions known to be involved in the opiate withdrawal syndrome in the adult rat; the amygdala, the periaqueductal gray (PAG), and the locus ceruleus. Injections of any of several doses of methylnaloxonium into the locus ceruleus and PAG elicited physical signs of opiate withdrawal with subtle differences between the two sites. Injections of methylnaloxonium into the amygdala produced no behavioral change. Although physical signs of withdrawal from the amygdala in the adult are substantially milder than the PAG or LC, they do occur in the adult rat.

In conclusion, it seems likely that the same neural circuitry mediates physical withdrawal at different ages, regardless of the specifics of the behaviors elicited. These results suggest that the neural circuitry that initiates withdrawal matures early, but "down-stream" effector systems are later developing.

ACKNOWLEDGMENTS

I wish to thank Dr. Gordon A.Barr, who has been and will continue to be my mentor. I would like to thank him for his patience and guidance in allowing me to pursue this dissertation project. I would also like to thank the members of my committee, Dr. Vicky Luine, Dr. Gerald Turkewitz, Dr. Eliot Gardner, and Dr. Cynthia Kuhn for their helpful suggestions and support. A very special thanks to Dr. Herbert Krauss for taking a chance on me and providing me with much needed encouragement and kindness. I would also like to thank Dr. Peter Moller for his guidance throughout my years in the Biopsychology program.

I am very grateful to Duckhyun Kim Yi, my dear friend and co-worker, who made life in graduate school much more enjoyable for me and provided me with some very fond memories and a life long friend. I also wish to thank other members of the lab, Melissa Klein, Jean Willi, and Rebecca Smart for their help and friendship. I am grateful to Shaoning Wang for his assistance in data collection in the early stages of this project and to Ellen Breheny for her technical assistance.

Completing a task such as this is much easier with the help of close friends and family. I am very grateful to my close friend Lisa D'Augostine who has always

been there for me with constant words of encouragement and friendship. I would also like to thank my brother, Dr. Glen E. Jones for his support of this endeavor.

Most of all I wish to thank my parents who have taught me the true meaning of dedication. I will forever be grateful for their love and support. Therefore, I wish to dedicate this work to my parents, Ronny and June Jones.

This work was supported in part by grant DA 06600.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1
General Introduction

Opioid compounds include endogenous peptides and their synthetic analogues, alkaloids derived from opium such as morphine and codeine, and semisynthetic alkaloids such as heroin and methadone. These compounds interact with several closely related receptors and modulate a variety of physiological functions, such as nociception, hormone secretion, neurotransmitter release, feeding, respiratory depression, gastrointestinal motility, and opiate addiction (Chen, et al., 1993; Pasternak, 1988). Chronic exposure to opiate drugs results in stereotypical behaviors associated with drug addiction, including tolerance and physical dependence. Tolerance is a term applied to the increasing insensitivity of the central nervous system (CNS) to the effects of any drug. Physical dependence is usually defined as the expression of "intense physical disturbances" upon removal of the drug after chronic administration of an opiate (Maldonado, et al., 1992). Physical dependence on opiates is characterized by abstinence behaviors when opiate intake is abruptly terminated or when an opiate antagonist is administered. The opiate withdrawal syndrome is an integral part of the opiate addictive process and has been so extensively studied that it is considered to be the prototypic measure of opiate dependence.

Opiate withdrawal symptoms in the human adult

During the withdrawal syndrome the CNS will experience a period of

readaptation as it resumes normal function normally in the absence of the opiate drug. It is during this time that the individual will experience physical signs of withdrawal. The signs and symptoms of opiate withdrawal have been associated with both physiological and behavioral responses that reflect actions opposite to those of the acute effects of opiates. Since the acute effects of opiates are generally depressant, withdrawal results in a hyperexcitable state. According to Jaffe (1990), in the adult, symptoms such as lacrimation, rhinorrhea, yawning, and sweating will appear around 8 to 12 hours after the last dose. As the syndrome progresses, additional signs and symptoms appear such as dilated pupils, restlessness, irritability, and tremor. As the syndrome reaches its peak (48 to 72 hours) the withdrawing individual will exhibit insomnia, marked anorexia, violent yawning, and sneezing, with weakness and depression becoming pronounced (Haertzen & Hooks, 1969; Henningfield, et al., 1987; Jaffe, 1990). The withdrawing individual will fluctuate between chills and excessive sweating. Nausea, vomiting, diarrhea, and abdominal cramps are common as well as increases in heart rate and blood pressure. Other signs of hyperexcitability include ejaculation in men and orgasm in women (Jaffe, & Martin, 1980). Furthermore, cues associated with this syndrome in the human adult are responded to as noxious conditioned stimuli (O'Brien, et al., 1988). At any point in the course of

withdrawal, the administration of a suitable opiate will suppress the symptoms of withdrawal. In the absence of treatment, most of the observable symptoms will disappear within 7 to 10 days (Jaffe, & Martin, 1980; Jaffe, 1990). However it is not clear how long it takes to restore physiological equilibrium.

Opiate withdrawal in the human infant

Opiate drugs cross the placenta in the pregnant women, passively exposing the fetus to these drugs if they are used during pregnancy (Kirby, 1981a). Exposure to opiate drugs during pregnancy lead to fetal and neonatal death, spontaneous abortions, premature delivery, fetal aspiration, a reduction in somatic and brain weight as well as subtle behavioral abnormalities (Chasnoff, 1988). An important correlate to opiate exposure in the immature animal is the development of physical dependence in the fetus and neonate. In the neonate this syndrome is precipitated by the abrupt removal of the drug following delivery. At birth, the newborn will continue to metabolize and excrete the drug. Following birth, as they are no longer able to absorb the drugs from the mother's blood, the withdrawal behaviors will begin to appear. The neonatal abstinence syndrome (NAS) in the human infant is characterized by central nervous system problems and dysfunctions of the autonomic system. These symptoms include gastrointestinal disturbance, respiratory distress, yawning, sneezing, fever, high-pitched crying, increased

muscle tone, and irritability. Although these infants suck their fists frantically, they have difficulty with feeding due to an uncoordinated sucking reflex. Further investigation of the infants of drug-addicted mothers has also demonstrated additional signs such as a variety of endocrine abnormalities (Dube, 1981), altered evoked potential to auditory and visual stimuli (Neumann, & Cohen, 1978), increased frequency of rapid eye movement, poor organization of sleep states (Dinges, et al., 1980), and a decreased head circumference. Approximately 80 to 90 percent of infants passively exposed to opiates through maternal ingestion during pregnancy will manifest these symptoms ranging from mild to severe (Franck, & Vilardi, 1995). The onset of these symptoms will vary in individual infants, with the majority of symptoms arising within 72 hours (Desmond & Wilson, 1975; Finnegan, 1976; Jaffe, 1990; Kaltenbach, 1994). The onset of withdrawal is dependent on the following factors: (1) time since last dose, (2) amount and strength of the drug, (3) duration/extent of maternal use, (4) drug half-life (The half-life for heroin in the newborn is 4 hours; for methadone, it is 23 hours), (5) effects of maternal poly-drug use in combination with drugs used during labor and delivery, and (5) metabolism and excretion of the drug by the neonate (Franck, & Vilardi, 1995; Hodding, et al., 1980). Although follow-up investigations on the opiate-exposed infant are few, there are indications of

persistent effects of the drugs beyond the perinatal period. Early symptoms such as diarrhea, irregular sleep patterns and irritability may continue in attenuated form for up to 6 months after birth (Desmond & Wilson, 1975).

Opiate withdrawal in the human fetus

Although these symptoms have been well defined in the human infant, the literature is scarce regarding withdrawal from opiates in the fetus. Opiates may adversely affect the fetus by compromising the delivery of oxygen and substrates to the fetus (Szeto, 1995). The respiratory depressant actions of opiates in humans and animals are well known and may decrease fetal oxygen availability. The placentas of heroin-exposed infants commonly reveal meconium histiocytosis, indicating that they experienced hypoxia or some other form of distress during fetal life, perhaps related to episodes of heroin withdrawal (Finnegan, 1976). It has also been suggested that periodic episodes of heroin withdrawal during pregnancy might restrict fetal growth by reducing uterine or placental bloodflow (Naeye, 1965). Any reduction in uteroplacental perfusion coupled with respiratory depression may significantly reduce the delivery of oxygen and substrates to the fetus (Szeto, 1995). Fetal hypoxia and hypoglycemia are known to alter fetal cardiorespiratory and neurobehavioral function. Acute hypoxia has been shown to cause fetal bradycardia and decrease rapid eye movement (REM)

sleep and fetal breathing activity (Boddy et al., 1974; Koos, et al., 1987). Fetal hypoglycemia has also been shown to significantly alter fetal EEG and decrease breathing activity (Richardson, et al., 1982). Due to the fact that opiates do cross the placental barrier and since drug use among pregnant women is often characterized by repeated exposure and withdrawal it is likely that the fetus also experiences withdrawal *in utero*. Some of the deleterious effects observed in infants exposed to opiates during pregnancy may therefore be a consequence of the fetus experiencing opiate withdrawal *in utero*.

Opiate withdrawal in the adult rat

In the human, the complexity of the issues associated with drug seeking behavior in the pregnant woman makes it impossible to determine exactly what aspects of the syndrome are the direct result of opiate abuse. For example, opiate addicted mothers often abuse other drugs, suffer from malnutrition and usually have a low socio-economic status. Unfortunately, until very recently the developing animal has not been a model used to examine the adverse effects of opiates. This is probably because the symptoms associated with opiate withdrawal in the young animal are subtle and differ from those observed in the adult animal which has served as a primary paradigm in drug abuse research. The animal model most commonly used in the study of opiate withdrawal is the adult rat. The opiate

withdrawal syndrome in the adult rat has been well described behaviorally, anatomically, and most recently at the molecular level. The abrupt termination of chronic morphine treatment (spontaneous withdrawal) or administration of an opiate antagonist (precipitated withdrawal) the adult rat results in a complex syndrome composed of both stereotyped behavioral and autonomic symptoms which include wet dog shakes, increased activation, jumping, teeth chattering, diarrhea, ptosis, weight loss, hypothermia and will emit both low and high frequency ultrasounds. The physiological changes are characteristic of sympathetic discharge, and include increased arterial blood pressure, adrenal and lumbar sympathetic activity, and plasma catecholamines levels. A strong aversive component to opiate abstinence has also been suggested. Adult rats suppress drinking fluids and learn taste aversions (Koob, et al. 1989; Mannering & Jackson, 1977; Pilcher & Stolerman, 1976), decrease electrical self-stimulation rates (Glick & Charap, 1973; Jackler et al., 1979; Shaefer & Michael, 1983), avoid entering places (Mucha et al., 1982; Mucha, 1987), and bury small objects to which they have been exposed during precipitated withdrawal (Mucha, 1991).

Anatomy of opiate withdrawal in the adult rat

The anatomical sites that mediate these diverse symptoms of opiate withdrawal in the adult rat have been explored using many different methods

including lesions of specific nuclei to block withdrawal, measurements of regional cerebral glucose utilization (RCGU), and intracerebral injections of morphine and morphine antagonists. The expression of the c-fos proto-oncogene has also served as a tool in identifying the anatomical structures involved in opiate withdrawal. Lesions of the medial forebrain bundle, or of the ventromedial nuclei of the hypothalamus, that interrupt this bundle, reduced several signs of opiate abstinence (Glick & Charap, 1973; Kerr & Pozuelo, 1971). Thalamic, limbic, and cortical lesions did not alter the withdrawal syndrome (Adler et al., 1978). Lesion of the central amygdala or a combined transection of the stria terminalis and ventral amygdalafugal pathway blocked the jumping behavior of the dependent animal without affecting the remaining signs (Calvino, 1979). Large lesions of the locus coeruleus reduced chewing, rearing, piloerection, and hyperactivity induced by ICV injection of the opiate antagonist methylnaloxonium (Maldonado & Koob, 1993). RCGU studies have demonstrated elevated glucose use at multiple sites, including several cortical areas, many hypothalamic nuclei, limbic structures such as the nucleus accumbens (nAcc), diagonal band of Broca, septum, amygdala, and the hippocampus, basal ganglia, midbrain structures, notably the PAG, raphe nuclei, medial and lateral habenula, the LC and thalamus (Adams & Wooten, 1990; Geary & Wooten, 1983, 1985a, 1986; Kimes & London, 1989). C-fos expression

reveals involvement in similar brain sites. It should also be noted that the distribution and magnitude of elevated metabolic activity is similar in both spontaneous withdrawal and precipitated withdrawal, occurring primarily in limbic and limbic related structures (Geary & Wooten, 1985b). RCGU analysis further reveals that changes in cerebral metabolism are almost identical in limbic and brainstem structures during opiate withdrawal whether the opiate dependence was induced by the ICV or subcutaneous route of administration (Adams & Wooten, 1990). Precipitating withdrawal by intracerebral injections of opiate antagonists is perhaps the most direct method to assess the brain structures involved in specific withdrawal behaviors. Areas found to be highly sensitive to the opiate antagonist naloxone are the medial hypothalamus, the periaqueductal gray (PAG), amygdala, medial thalamus, and globus pallidus (Tremblay, & Charton, 1981). Since naloxone is a lipophilic opiate antagonist it is possible that these studies involved a rapid diffusion of the drug. More recent studies have utilized methylnaloxonium, a hydrophilic opiate antagonist, in order to limit the spread of the drug within the brain (Maldonado et al, 1992; Schroeder et al., 1991). The most sensitive site for methylnaloxonium-precipitated withdrawal is the locus coeruleus (LC). Injections of methylnaloxonium into this area elicit signs such as jumping, rearing, chewing, and locomotor activity. Rearing and locomotor activity are also greatly increased

following methylnaloxonium administration into the PAG while wet dog shakes are elicited with injections into the anterior preoptic hypothalamus and nucleus raphe magnus. Methylnaloxonium injections into the amygdala produces a weak withdrawal syndrome, eliciting fewer of the physical signs (Maldonado et al., 1992). These data indicate that while the locus coeruleus, and the periaqueductal gray matter play an important role in eliciting some of the physical signs of withdrawal, no one single brain structure is entirely responsible for all of the signs that are associated with the opiate withdrawal syndrome in the adult rat. Furtherfmore, chronic administration of opiates into the brain show that anatomically distinct opiate receptor fields mediate different components of addiction such as reward and physical dependence. For example, rats never before exposed to opiates rapidly learn to press a lever for microinjection of morphine into the ventral tegmental area. Challenge by a narcotic antagonist produced no signs of physical dependence. Dependence was not seen after long-term morphine infusions into the ventral tegmentum but was seen after similar infusions into the PAG (Bozarth & Wise, 1984)

Cellular and molecular mechanisms of opiate withdrawal in the adult

Following the discovery of opioid receptors (Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973) and endogenous opioid peptides (Goldstein et al.,

1979), researchers began to speculate that the opiate addiction process in the adult rat might be mediated by the dysregulation of the expression of opioid receptors or opioid peptides. However, chronic treatment with opiates, in paradigms which produce physical dependence has failed to produce consistent changes in opioid receptor number. While some groups have shown that chronic exposure of adult animals to morphine does not affect the density of mu opioid receptors (De Vries, et al., 1993; Abdelhamid, & Takemori, 1991; Nishino, et al., 1990), others have reported either upregulation (Brady, et al., 1989) or downregulation of opiate receptors (Bhagrava, & Gulati, 1990; Tao, et al., 1990). This lack of consistency in findings in terms of neurotransmitters and receptors has shifted attention to the role of postreceptor mechanisms. Recent findings have demonstrated that changes in the activity of G-proteins and the cAMP second messenger and protein phosphorylation pathways mediate important aspects of opiate dependence (Nestler, 1992). Data are now available in the adult rat that demonstrate how behavioral, cellular and molecular mechanisms interact together to produce the phenomenon of opiate withdrawal.

Opioid receptors have been divided into at least three main classes: κ, μ, and σ, as well as several subclasses. These receptors differ in their affinity for various opioid ligands and in their cellular distribution, although there is overlap of

function as well as distribution. With respect to ligands, the σ receptors have the highest affinity for enkephalins, the κ receptors potentially bind dynorphins, and the μ opioid receptors are sensitive to morphine and some of its alkaloid analogs (Yasuda, et al., 1993; Wang, & Lovick, 1993). In the adult animal, the development of morphine dependence is controlled not only by μ-receptors, but also by σ, and κ-receptors. These receptors appear to have opposite influences on the development of dependence (Yukhananov, et al., 1994). The selective κ - opioid antagonist, norbinaltorphimine, significantly increases the level of tolerance to morphine (Sofuoglu, et al., 1992), whereas the κ-agonist, U-50, 488, blocked the development of tolerance and attenuated, naloxone precipitated physical withdrawal in morphine treated animals. The selective antagonists of σ-receptors, naltrindole and naltrindole 5'-iosthiocyanate, have been found to attenuate the level of tolerance to morphine and reduced or eliminated the physical signs of naloxone precipitated morphine withdrawal (Abdelhamid, et al., 1991).

The role of opioid receptors in fetal and neonatal withdrawal are still unclear. Binding studies have detected opioid receptors in the rat brain by the 14th day of gestation (Clendeninn, et al., 1976). Autoradiographic studies have indicated that μ and κ receptors are present at significant densities during early neonatal periods in several brain regions (Kornblum, et al., 1987), while σ

receptors are absent until the 2nd postnatal week (Spain et al., 1985). Physiological studies have provided evidence that the opiate system is functional and has the capacity to affect fetal behavior *in utero*. For example, exogenous morphine administration to the pregnant female has been shown to depress nonevoked activity of fetuses *in utero* (Kirby, 1981a). Smotherman & Robinson (1992) have shown that milk engages the endogenous opioid system of the fetal rat and affects fetal responsiveness by interacting with the κ receptors of the opioid system. These studies taken together argue that the opioid receptors are present during the fetal period and the exogenous manipulations can exert effects on fetal behavior. It seems unlikely that the σ receptors have a significant role in the physical signs of withdrawal until after the 2nd week of life. However, since μ and κ receptors are present and functional in the fetus and neonate, it is likely that they contribute to the withdrawal syndrome of the infant.

The recent cloning of the opioid receptors (Evans, et al., 1992; Kieffer, et al., 1992, Meng, et al., 1993; Yasuda, et al., 1993; Thompson, et al., 1993) suggest that they belong to a family of membrane receptors that transduce their intracellular signals via G-protein-coupled receptors (Childers,1993; Loh, & Smith, 1990; Cox, 1993). The opioid receptors are coupled to second messenger systems by guanosine nucleotide-binding protein, which are also known as G proteins

(Kandel & Swartz, 1991). Normally, when a neurotransmitter is bound, the receptor interacts with the G protein, producing a conformational change that results in either an activation or inhibition of appropriate ion channels or second messengers. Initially, when opiate administration is begun, the opioid receptors are coupled to G proteins. A functional decoupling of opioid receptors from G proteins will occur as a result of chronic opioid receptor occupation (Christie et al., 1987; Trujillo & Akil, 1991). This will cause the acute actions of the drug to diminish. Thus tolerance develops as higher doses of the opiate are necessary to trigger a second messenger response that will produce both physiological and behavioral change. It has been previously proposed that the biosynthesis of endogenous peptides will also decrease during chronic exposure to opiates (Trujillo, & Akil, 1991). This view postulates that when the administration of the opiate is terminated, initially the endogenous peptides will not be available to compensate for the loss of the exogenous opiates. When the exogenous drug is not available to act on the receptors, a rebound hyperexcitability of opiate-responsive neurons occurs (Trujillo, & Aklil, 1991). It is during this time that the signs and symptoms of opiate withdrawal will be manifest. The withdrawal syndrome will not subside until endogenous peptides become available and receptor coupling return to normal. At present there is no available data to

support this theory.

The cloning of the opioid receptors has further allowed for an investigation into our understanding of how alterations in their gene expression and protein structures contribute to the development of drug addiction. Cellular adaptation to opiates, reflected in the regulation of the expression of many different mRNAs, seems likely to contribute to behaviors associated with addiction (Mackler, & Eberwine, 1994). For example, after opioid stimulation or withdrawal, the abundances of several mRNAs are changed in discrete brain regions, including mRNAs for c-fos in the stiatum (Chang, et al., 1988), tyrosine hydroxylase in the locus coeruleus (Guitart, & Nestler, 1990), and vasopressin and other neuropeptides in the hypothalamus and striatum. Furthermore, precipitated withdrawal by naloxone also produces changes in the relative amounts of several mRNA molecules (Mackler, & Eberwine, 1994). Precipitated withdrawal with naloxone produces a decrease in K+ channel mRNAs while increasing CA2+ and NA+ channels (Mackler, & Eberwine, 1994). The effects of these increases in CA2+ and NA+ channels on neuronal activity provides a possible mechanism to explain neural hyperexcitability during withdrawal from opioids. For example, according to Mackler (1994), hyperexcitability may occur by lowering the threshold for the initiation of action potentials in excitable cells that contain more

voltage-sensitive Na+ or Ca2+ channels.

The locus coeruleus (LC) noradrenergic system of the adult rat has served as a useful model in describing the molecular mechanisms underlying the opiate withdrawal syndrome. The locus coeruleus is a major noradrenergic nucleus located on the floor of the fourth ventricle in the anterior pons and possesses a high density of opioid receptors, particularly of the μ and κ type (Tempel, & Zukin, 1987). A noradrenergic hyperactivity in the LC has been hypothesized to mediate the expression of some components of the morphine withdrawal syndrome and several neuroanatomical, neurophysiological and biochemical studies support this hypothesis. For example, an increase in the noradrenergic neuron firing rate in the LC has been reported during naloxone-precipitated morphine withdrawal that shows a similar time course to that of the behavioral signs of withdrawal (Aghajanian, 1978), the LC is the most sensitive site for the induction of withdrawal signs by intracerebral injections of methylnaloxonium (Maldonado, et al., 1992), destruction of the LC decreases physical signs of opiate withdrawal, (Maldonado, & Koob, 1993), and local infusions of the α2-adrenoceptor agonist clonidine, which suppresses withdrawal-induced activation of LC neurons (Aghajanian, 1978), attenuates both the behavioral (Taylor, et a., 1988), and biochemical changes associated with opiate withdrawal (Laverty, & Roth, 1980).

Futhermore, stimulation of the LC produces several behavioral and physiological signs of morphine withdrawal (Rasmussen, et al., 1990).

It has also been demonstrated that opiate administration leads to a dramatic upregulation of the cAMP (second messenger) system in the LC (Nestler, 1992). It has been proposed that such an up-regulated G-protein/cAMP system contributes to opiate withdrawal in the LC (Nestler, 1990). Rasmussen, et al., (1990), suggest that this up-regulated cAMP system could contribute to the activation of the LC seen during the early stages of opiate withdrawal. This group postulates that since opiates are known to inhibit LC neurons acutely, the up-regulated cAMP system observed in opiate-dependent animals could represent a "homeostatic" response to persistent opiate inhibition of cells. This view suggest that the concurrent presence of morphine and the up-regulated cAMP system in opiate-dependent animals would result in LC firing rates close to control levels. When the morphine is withdrawn abruptly by administration of naltrexone, the up-regulated cAMP system, unopposed by morphine, would thereby increase the activity of the LC neurons (Rasmussen, et al., 1990; Nestler, 1992). This model is supported by the finding that the time course by components of the cAMP system recover during opiate withdrawal follows the early, rapid phase of withdrawal recovery during which time LC neuronal firing rates begin to decrease

(Rasmussen, et al., 1990). An up-regulated cAMP system may therefore in part contribute to the early withdrawal activation of neurons within the LC.

On the other hand, there is increasing evidence that suggests that withdrawal-induced hyperactivity of LC neurons is not induced by altered intracoerulear mechanisms. This is strongly supported by the fact that even though increased firing rates of LC neurons in vivo were correlated with opiate withdrawal precipitated by systemic injections of naloxone, little or no activation was reported following application of naloxone directly into the nucleus of dependent animals (Aghajanian, 1978; Akaoka, & Aston-Jones, 1991). Moreover, no withdrawal activation was observed during opiate withdrawal in LC neurons recorded in the *in vitro* slice (Andrade, et al., 1983; Christie, et al., 1987). A lack of change in firing of LC neurons in the slice preparation *in vitro* during opiate withdrawal suggests that the primary sites responsible for the dramatic increase in activity of these neurons may be afferent inputs into the LC (Andrade, et al., 1983). Recent data showing that intracerebroventricular or intracoerulear injections of excitatory amino acids (EAAs) substantially attenuated the activation of LC neurons induced by intravenous naloxone-precipitated withdrawal, suggest that an important part of hyperactivity in the LC during opiate withdrawal may be mediated by an excitatory amino acid input to the region of the LC (Guyenet, &

Young, 1987; Akaoka, & Aston-Jones, 1991). Since it has been shown that lesions to the area of the nucleus paragigantocellularis (Pgi) attenuates withdrawal-induced activation of LC neurons, and since the Pgi provides excitatory amino acid input to the LC (Ennis & Aston-Jones, 1988), it has been hypothesized that this nucleus may be responsible for mediating the increased activation of LC neurons precipitated by sudden opiate withdrawal (Akaoka, & Aston-Jones, 1991).

Taken together these studies indicate that the expression of opiate withdrawal in the adult rat involves a combination of intrinsic LC activity as well as extrinsic inputs to the LC. These findings represent important advances in the understanding of the biochemical and molecular basis of opiate addiction.

Rationale for study

The initial characterization of the qualitative and quantitative aspects of behaviors associated with the opiate withdrawal syndrome in the adult rat paved the way for a more complete understanding of the neural mechanisms involved at the anatomical and molecular level. A complete understanding of the anatomical, biochemical, and molecular mechanisms that mediate behaviors associated with drug addiction and dependence have important clinical implications. A clearer understanding of these mechanisms will increase the likelihood of the development of pharmacological agents that might possibly prevent or reverse the actions of

opiates on specific target neurons as well as treat the physical symptoms associated with the opiate withdrawal syndrome.

The existence of a fetal and neonatal withdrawal syndrome has only been hypothesized in animal models based on teratologic studies (Kuwahara & Sparber, 1981; Lichtblau, & Sparber, 1981). It has also been shown that chronic exposure to morphine causes a decrease in fetal activity that is reversed by naloxone (Kirby, 1981a), suggesting the existence of an opiate withdrawal syndrome *in utero*. In the infant rat, ultrasonic vocalization levels are increased following spontaneous or precipitated withdrawal (Barr, & Wang, 1992). Previous studies have supported the argument that key components of a functional opioid system, including endogenous opioids and opioid receptors, are present at birth as well during the prenatal period (Spain, et al., 1985; Kirby, 1981a). Yet there is no detailed description that profiles the opiate withdrawal syndrome in the developing animal. Perhaps the absence of adult-like physical withdrawal signs in the infant rat has contributed to the lack of research regarding the withdrawal syndrome in the developing animal. Taking into account obvious developmental restraints in the very young animal, it seems likely that an opiate withdrawal syndrome would manifest itself in behaviors that are within the animals capacity to perform. Since it is likely that the withdrawal behaviors associated with the developing animal are

different from those observed in the adult animal, there is also no reason to assume that the underlying mechanisms that mediate the withdrawal syndrome are the same.

The hypothesis of this theses is that both the fetus and infant animal are capable of experiencing opiate withdrawal when the age specific behavioral repertoire appropriate to the age of the animal is examined. The goal of this dissertation project is therefore to describe in detail the unconditioned behavioral changes that follow precipitated withdrawal from chronic exposure to morphine during development, including the fetus, the neonate, and continuing through adolescence and to define specific anatomical mechanisms that mediate the withdrawal syndrome in the infant rat. This is a 2-stage process. In the first stage, the dam or the rat pup is treated chronically with morphine. Injections of naltrexone will be used to precipitate withdrawal. Behavioral changes will be assessed from fetal age through adolescence. In the second stage, methylnaltrexonium will be microinjected into one of three different brain structures in morphine dependent infant animals. These sites will include the amygdala, the periaqueductal gray (PAG); and the locus ceruleus. These sites have been chosen because they are sites invovled in the morphine withdrawal syndrome in the adult rat. In the adult rat, the most sensitive site for precipitated

methylnaloxonium-precipitated withdrawal is the LC, eliciting signs such as jumping, rearing, teeth chattering, chewing, and locomotor activity (Maldonado, et al., 1992). Likewise, the administration of methylnaloxonium into the PAG of adult rats also elicits a severe withdrawal syndrome with rearing and hyperactivity particularly elevated. The injection of methylnaloxonium into the amygdala in adult rats induced only a very mild withdrawal in which teeth chattering and chewing were observed (Maldonado, et al., 1992). Furthermore, autoradiographic studies show that these structures are densely labeled with opioid binding.

Chapter 2

The Ontogeny of Morphine Withdrawal in the Postnatal Rat

Introduction

Many pregnant women are exposed to opiate drugs such as heroin and methadone. Infants born to these women have a higher incidence of morbidity and mortality than do the offspring of nonaddicted women. It is a general assumption that perinatal morbidity is caused by the development of narcotic dependence in the fetus as a direct result of placental transport of the drug. However, these difficulties may have their origin *in utero* as the result of the fetus experiencing withdrawal during gestation (Lichtblau and Sparber, 1981; Kuwahara and Sparber, 1981). At birth, the newborn infant continues to experience withdrawal due to the absence of the drug. The opiate withdrawal experienced by these infants represents a crucial aspect of the opiate addictive process. The human infant, passively exposed to opiates *in utero* through the therapeutic use of methadone, the illicit use of heroin, or postnatally through breast feeding, demonstrates characteristics of withdrawal including irritability, a high-pitched cry, excessive sucking of fingers, sneezing, yawning, excessive weight loss, difficulty in regulating state, sleep and gastrointestinal disturbances (Finnegan, 1976). The withdrawal syndrome in the human infant is perhaps better described than for non-human species. However, it is difficult to obtain an accurate assessment of the withdrawal signs unique to infancy due to the influence of such factors as the

quality of prenatal care, nutrition, and polydrug use.

The animal model most commonly used by investigators to study the opiate withdrawal syndrome is the adult rat and that syndrome has been described in detail. Morphine dependent adult rats exhibit similar withdrawal symptoms when morphine intake is abruptly terminated (spontaneous) or an opioid antagonist injected systemically (precipitated). Symptoms following spontaneous or precipitated withdrawal in the adult rat include autonomic signs such as diarrhea, ear blanching, ptosis, exopathalamos, salivation, rhinorrhea, lacrymation, and penile erection/ejaculation, and motoric signs such as rearing, jumping, writhing, wet shakes, exploring, and abnormal posture. On the other hand, the behavioral and physiological consequences associated with opiate withdrawal in infant animals are not well defined. This is in part because withdrawal in infants may be considerably more subtle. For example, the autonomic changes that exemplify the adult abstinence syndrome are less severe in the neonate (Faneslow & Cramer, 1988). One possible explanation for this is that the physiological systems that mediate the adult signs of opiate withdrawal are not fully developed in the infant rat. Therefore, the absence of adult-like withdrawal behaviors alone should not lead to the assumption that the infant rat does not undergo morphine withdrawal but rather it suggests that morphine abstinent rat pups may demonstrate

withdrawal behaviors that are more appropriate for their age. For example, separation induced ultrasonic vocalizations (crying in response to separation from the familiar cues of the home environment) increase following naltrexone treatment in both methadone and morphine exposed pups at 7 days of age (Barr & Wang, 1992; Zmitrovich, et al., 1993) as they do in the adult (Vivian & Miczek, 1991). Therefore, it is our hypothesis that the infant animal may experience opiate withdrawal when the specific repertoire appropriate to the age of the animal is examined. In order to provide a developmental description of how the opiate withdrawal syndrome may change throughout development, we precipitated withdrawal in morphine dependent 7, 14, 21, and 42 day old rats and measured the resultant change in behavior.

Method

Subjects

The subjects were offspring of Long-Evans hooded rats mated in our laboratory. The parent animals were housed in plastic tubs in a colony room maintained at 22-24°C with a 12 h light/12h dark photocycle with light onset at 7 AM. Cages were checked twice daily, at approximately 10 AM and 7 PM. Pups found at either time were termed 0 days of age. After parturition, litters were culled to 8 pups without regard for the ratio of males to females. animals were

weaned at 21 days of age.

Design and Procedures

Animals were tested at 7, 14, 21, and 42 days of age. On the first treatment day (which differed for each group), the litter was removed from the dam (except for 42 day-old rats who were housed separately) and individual rats were injected with morphine sulfate (10 mg/kg, i.p.) twice daily (10 AM and 6 PM) for 6.5 days. The 7 day-old age group received one of two doses of morphine (3 mg/kg or 10 mg/kg, i.p., b.i.d.). Controls included saline injected pups (an untreated 7 day-old age group was tested to observe if the brief handling for saline injection might cause behavioral change). The last injection was on the morning of the 7th day. The order of the treatment conditions were assigned randomly with the exception that a maximum of 6 litters were used per condition. On the afternoon of the 7th day, animals were transported from the animal facility to our lab in plastic tubs with bedding and placed in an observation chamber maintained at approximately 33°C. One pup was weighed, rectal temperature recorded and injected with either saline, 0.3, 1.0, 3.0, or 10.0 mg/kg naltrexone i.p. to precipitate withdrawal. The doses were randomly assigned and the observer was blind to each dose. For the preweaning age groups, the pup that received the naltrexone (or saline) injection was then placed back into the observation chamber

with the remainder of the litter (without the dam). The pup was then observed for a total of 20 minutes and behaviors recorded every 15 seconds on a checklist. The checklist was developed in preliminary experiments by observing and recording behaviors displayed by morphine treated pups after an injection of naltrexone. Also incorporated into this checklist were behaviors cited in previous reports on opiate withdrawal in the adult rat (see Table 1 for definitions). Adult behaviors such as scratching, ear blanching, ptosis, and rhinorrhea were also measured but never occurred in our observations and are therefore not included in the behavioral definitions. When the observation period ended, rectal temperature was re-recorded and the pup was anesthetized and placed back into the litter. This was done in order to quiet the pup and prevent interference with the next observed pup. The second pup was then tested. A total of 5 pups from each litter were injected and tested in this manner. Postweaning rats were tested individually on fresh bedding in a chamber that measured 8.5 x 8.5 x 5 cm high and observed for 20 minutes in the same manner as preweaning pups.

Statistics

A factorial Analysis of Variance (ANOVA) was conducted for each

Table 1. Postnatal Behavioral Definitions

Burrow	sliding the body under the shavings of the observation chamber.
Diarrhea	watery stool
Grooming	licking any part of body including face washing.
Head Moves	lateral and rotary motions of the head.
Jumping	sudden leaping such that all four paws are off the bottom of the chamber.
Moving Paws	continuous movement of the hindpaws without walking.
Quiet	sedated appearance, with no movement.
Rearing	standing erect on two hind legs without leaning on wall.
Rolling	turning the body over at least one full rotation.
Separation	without contact with any litter-mates.
Stretching	extension or dorsal flexion of the trunk, causing an apparent lengthening of the body.
Teeth Chatter	movement of the jaw accompanied with constant chattering of the teeth.
Together	bodily contact with one or more litter-mates.
Walking	taking more than one step forward.
Wall Climbing	placing at least two forepaws on the wall of the observation Chamber.
Wet Dog Shakes	rapid shaking of the whole body.

behavior except for burrowing, diarrhea, jumps, and teeth chatter. These behaviors never occurred in the chronic saline treated animals. Therefore, an ANOVA was performed on morphine treated groups only for these behaviors. The 20 minute observation period was divided into 5 different time periods, each consisting of 4 minutes. Behaviors were summed in each time period which was treated as a within subjects variable. All doses were injected within a single litter and the drug dose effect was treated as a within subjects variable. The different chronic treatment groups and the different age groups served as between subjects variables.

Results

The results provide the picture of greatly increased activation, expressed by age specific behaviors, precipitated by naltrexone in morphine treated animals in all age groups. There were a total of seven possible effects: chronic treatment effects (morphine vs saline treated groups); age effects; interaction effects between chronic treatment and age; naltrexone dose effects (including the saline vehicle); interaction effects between dose and chronic treatment; interaction effects between dose and age; and interaction effects between naltrexone dose, chronic treatment, and age (see Table 2). The results also showed that three

Table 2. Results of ANOVA's for withdrawal behaviors

Behavior	*Ages Analyzed*	*Chr. Tx*	*Age*	*Chr.Tx*Age*	*Dose*	*Dose*Chr.Tx.*	*Dose*Age*	*Dose*Chr.Tx*Age*
Burrowing	21 & 42		F=4.31; ≤.067*		F=4.82; p≤.003		F=0.20; p≤.02	
Diarrhea	21 & 42		F= 5.20; p≤.05		F=2.25; p≤.08*		F = 2.32; p≤ .08*	
Grooming	14,21,&42	F=0.01; N.S.	F=44.25; p≤.0001	F=7.31; p≤.003	F=0.84; N.S.	F=2.24; p≤.07*	F=0.93; N.S.	F=2.06; p≤.04
Head Moves	all	F=85.42; p≤.0001	F=10.34; p≤.0001	F=9.65; p≤.0001	F=16.37; p≤.0001	F=13.84; p≤.0001	F=3.30; p≤.0001	F=4.36; p≤.0001
Jumps	14,21, & 42		F=14.84; p≤.0003		F=1.76; N.S.		F=1.41; N.S.	
Moving Paws	7 & 14	F=26.13; p≤.0001	F=0.62; N.S.	F=0.01; N.S.	F=9.30; p≤.0001	F=5.53; p≤.0006	F=1.00; N.S.	F=1.00; N.S.
Quiet	all	F=0.25; p≤.0001	F=10.65; p≤.0001	F=2.92; p≤.04	F=27.68; p≤.0001	F=23.64; p≤.0001	F=1.81; p≤.051*	F=1.62; p≤.09*
Rearing	21 & 42	F=1.46; N.S.	F=77.29; p≤.0001	F=1.89; N.S.	F=1.15; N.S.	F=0.90; N.S.	F=1.20; N.S.	F=1.03; N.S.
Rolling	7	F=6.74; p≤.001			F=5.45; p≤.001	F=2.10 p≤.02		
Separated	7,14,& 21	F=6.75; p≤.01	F=45.34; p≤.0001	F=0.70; N.S.	F=2.53; N.S.	F=0.60; N.S.	F=2.42; N.S.	F=0.84; N.S.
Stretching	all	F=5.37; p≤.003	F=6.27; p≤.01	F=4.07; p≤.01	F=1.50; N.S.	F=1.39; N.S.	F=1.75; N.S.	F=1.45; N.S.
Teeth Chatter	21 & 42		F=0.23 N.S.		F=0.26; p≤.05		F=0.88; N.S.	
Together	7,14, &21	F=7.12; p≤.01	F=3.91; p≤.0001	F=0.65; N.S.	F=2.70; p≤.03	F=0.66; N.S.	F=2.32; p≤.02	F=0.81; N.S.
Walking	all	F=58.05; p≤.0001	F=14.06; p≤.0001	F=0.67; N.S.	F=7.0; p≤.0001	F=7.17; p≤.0001	F=0.56; N.S.	F=1.06; N.S.
Wall Climbing	all	F=14.25; p≤.0005	F=40.39; p≤.0001	F=3.12; p≤.04	F=0.80; N.S.	F=1.85; N.S.	F=0.84; N.S.	F=0.51; N.S.
Wet Dog Shakes	14,21, & 42	F=34.91; p≤.0001	F=7.54; p≤.0024	F=6.18; p≤.006	F=6.51; p≤.0001	F=6.11; p≤.0002	F=2.33; p≤.02	F=2.08; p≤.04

Note- Burrowing, Diarrhea, Jumps, and teeth chatter did not occur in saline treated animals and therefore these behaviors were analyzed for the morphine treatment only. *Trend only.

different patterns of behavior occurred in animals undergoing precipitated withdrawal. These were divided into adult withdrawal behaviors, preweaning withdrawal behaviors, and withdrawal behaviors that were developmentally continuous.

Infant Withdrawal Behaviors

Head moves. This behavior was significantly increased in morphine treated groups as compared to control groups. Head moves were observed in all age groups, occurring more frequently in the morphine treated 7 and 14 day-old animals undergoing precipitated withdrawal. Within the 7 and 14 day-old morphine treated age group, the high numbers of head moves were seen in the 10 mg/kg morphine treated animals at the .3 mg/kg dose of naltrexone.

Moving Paws. This behavior was only seen in the 7 and 14 day-old age groups. Paw movement occurred significantly more often in morphine treated animals as compared to saline treated animals in both age groups. Paw movements were recorded frequently at all doses of naltrexone in the 7 day-old and 14 day-old morphine treated age groups.

Rolling. This behavior was unique to the 7 day-old age group. Rolling occurred significantly more often in both morphine treated groups, with all doses

above 0.3 mg/kg of naltrexone producing frequent amounts of rolling.

Stretching. Stretching was observed most frequently in the 7 day-old age groups. Within the 7 -old age group, the low morphine treated animals exhibited the most stretches. This behavior was rarely seen in 14 and 21 day-old animals, and was never observed in the 42 day-old animals.

Adult Withdrawal Behaviors

Burrowing. Burrowing only occurred in morphine treated animals in the 21 and 42 day-old age treated with naltrexone The most frequent number of observations were recorded in the 21 day-old morphine treated animals.

Diarrhea. Diarrhea only occurred rarely in morphine treated animals in both the 21 and 42-day old naltrexone treated animals, and was observed most frequently in the 42 day-old age group.

Jumps. Jumping was observed only rarely in the 14 and 21 day-old age groups. This behavior was recorded most frequently in the 42 day-old age group, in morphine treated animals, with the greatest amount of jumps elicited with the 10.0 mg/kg dose of naltrexone.

Teeth Chatter. This behavior was recorded for the first time in the 21 day-old age group. Teeth chattering was only observed in morphine treated animals, with equal amounts occurring in the naltrexone treated 21 and 42 day-old animals.

Wet Dog Shakes. This behavior was only observed in the 14, 21, and 42 day-old age groups. Wet dog shakes were significantly increased in morphine treated groups as compared to saline treated groups. Greater numbers of wet dog shakes were recorded in the morphine treated animals in the 21 & 42 day-old age groups.

Developmentally Continuous Withdrawal Behaviors

Quiet. Control animals were quiet significantly more often than the morphine treated animals in all age groups, with the dose response curve appearing similar at all ages.

Walking. Walking occurred in all age groups and was significantly increased by naltrexone in morphine treated animals as compared to saline treated animals.

Behaviors Demonstrating No Naltrexone Dose Effect

Grooming. Grooming was not observed in 7 day-old animals. The effect was age dependent. Morphine treated animals in the 21 day-old age group demonstrated less grooming than did the saline treated animals for this age group, but grooming occurred more frequently in the 42 day-old morphine treated animals as compared to the younger age groups, at least at the higher doses.

Separated. For this behavior there was a main effect of chronic treatment

(more separation occurred in the morphine treated animals as compared to saline treated animals) but there was no interaction of chronic treatment with either dose or age.

Together. Together showed a main effect for chronic treatment (animals were together less often in morphine treated groups as compared to saline treated groups) but there was no interaction of chronic treatment with either dose or age.

Wall Climbing. Wall climbing was observed in all age groups. There was no significant naltrexone effect although all morphine treated pups showed more wall climbing than did saline treated pups.

There were no differences found in pre and post-test temperatures between the different chronic treatment groups (see Table 3). Significant differences were observed in the weights among the different groups. Morphine treatment decreased body weight equally in each age group (see Table 4).

Figure 1. This figure depicts the mean number occurrences of infant behaviors (± one SEM) occurring mostly in the younger pups during naltrexone precipitated morphine withdrawal for each of four different ages of rats. Chronic treatment groups included morphine (10 mg/kg) and saline injected litters. In addition, the 7 day-old age group received one of two doses of morphine (10 mg/kg or 3 mg/kg). An additional untreated group was also added to this age group to observe if the brief handling for saline injection might cause behavioral change.

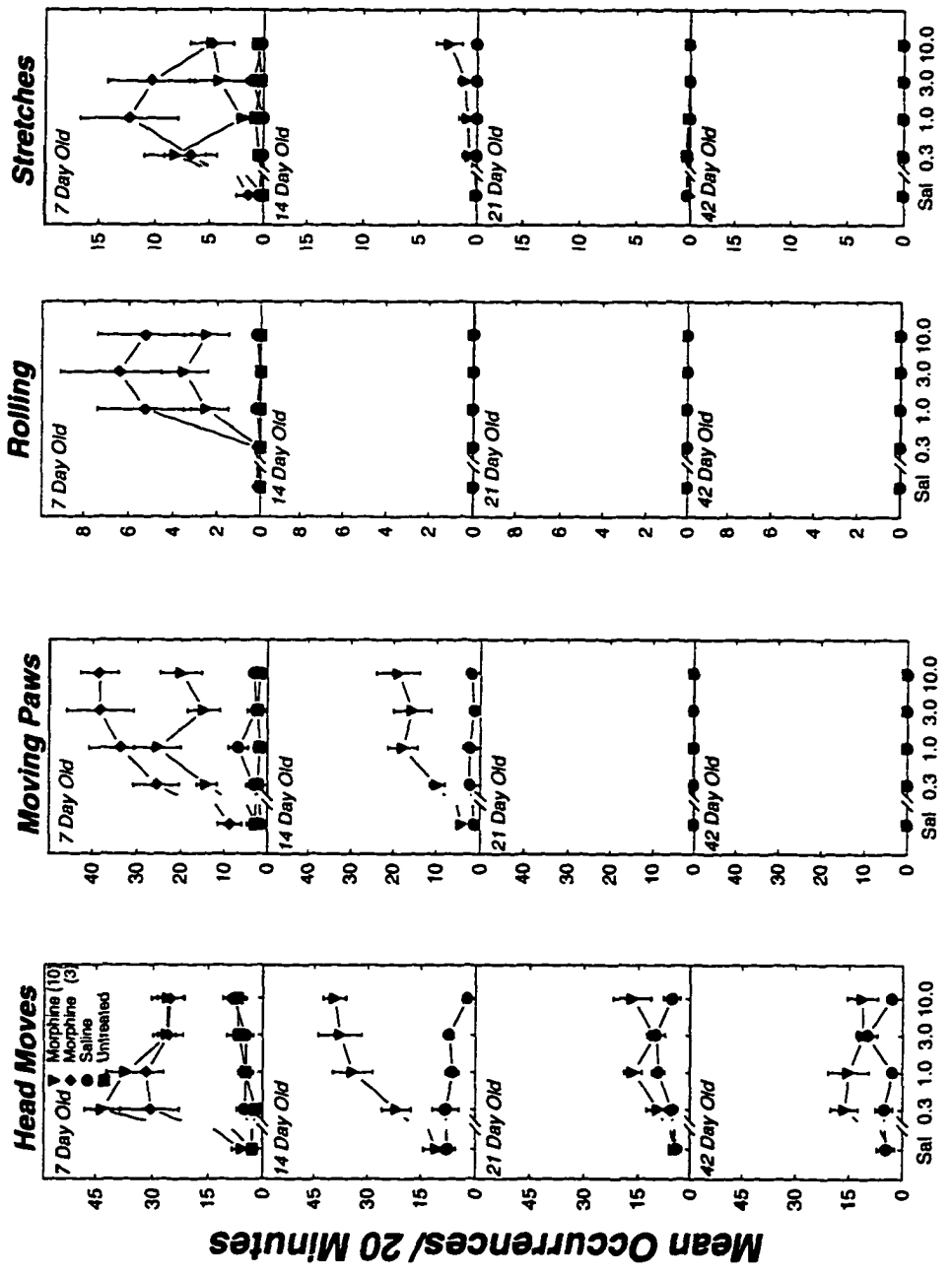

Figure 1

Figure 2. This figure depicts the mean number (± one SEM) of occurrences of withdrawal behaviors occurring mostly in the older animals during precipitated withdrawal for each different age group. Details are as described in the legend to the first figure.

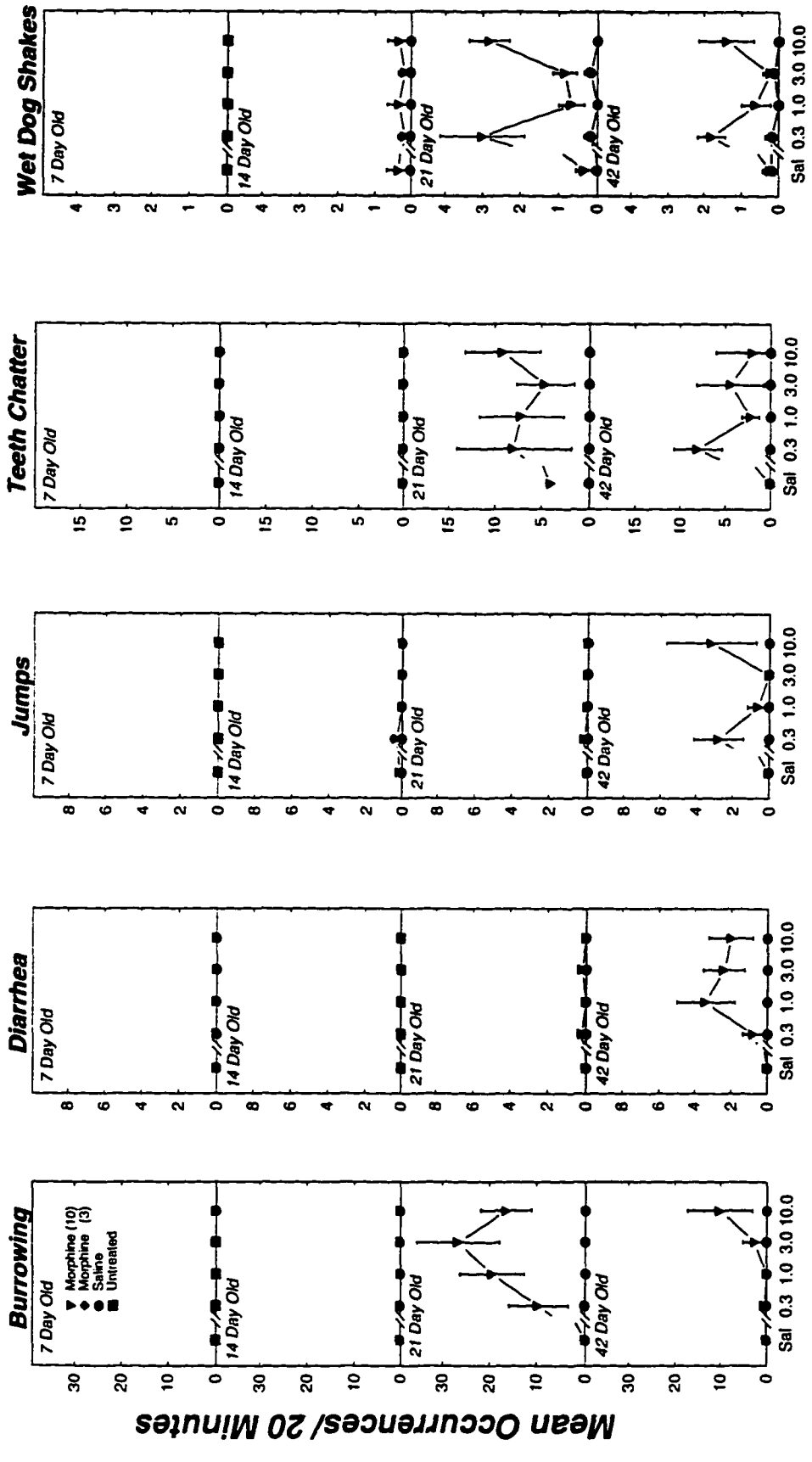

Figure 2

Figure 3. This figure depicts the mean number occurrences of precipitated morphine withdrawal behaviors that are seen at all ages studied. Details are as described in the legend to the first figure.

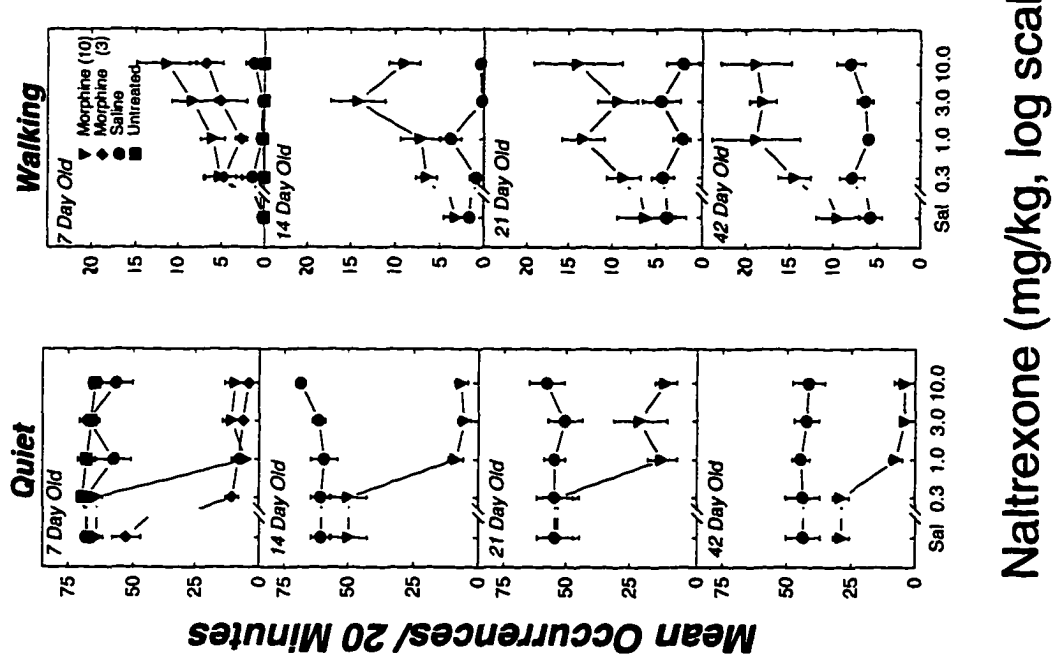

Figure 3

Figure 4. This figure depicts the mean number occurrences of behaviors for each age group that demonstrated no naltrexone dose effect. Details are as described in the legend to the first figure.

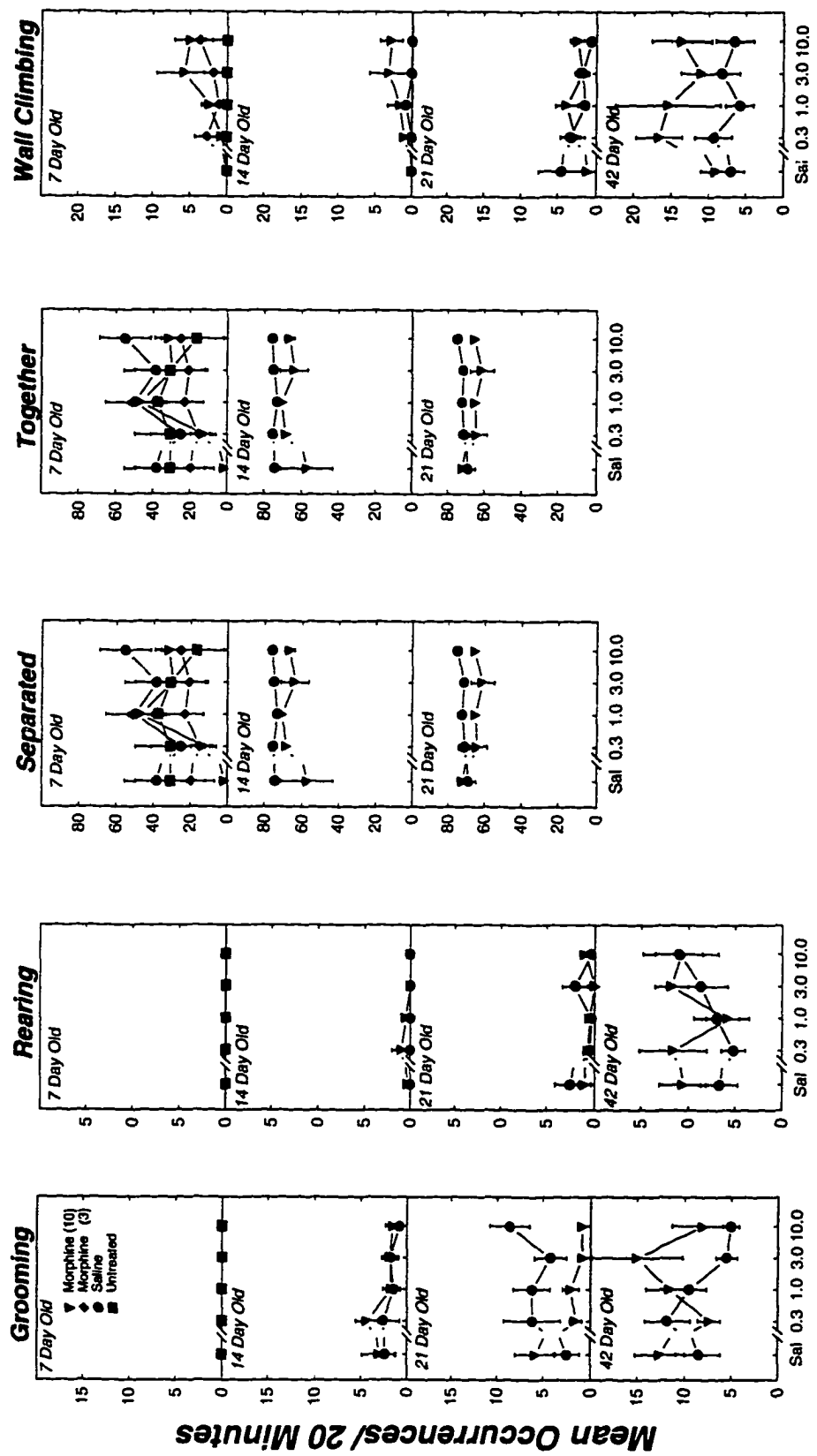

Figure 4

Table 4. Body Weight

Ages	*Saline Treated*	*Morphine Treated*
7 day-old	17.7± 2.2	13.0± 1.9
14 day-old	33.6± 4.7	27.9± 4.7
21 day-old	54.1± 4.8	44.0± 4.5
42 day-old	170.0± 9.2	158.2±14.3

Note : Cell entries are mean weights in grams ± one S.E.M. All pups within a litter were weighed and the weights averaged to provide a mean litter weight. Morphine treatment decreased body weight equally in each age group. N = 4 - 6 per cell.

Table 3. Body Temperature

	Saline					Morphine				
			Naltrexone					Naltrexone		
Ages	*0*	*0.3*	*1.0*	*3.0*	*10.0*	*0*	*0.3*	*1.0*	*3.0*	*10.0*
7 day-old	0.50±0.81	0.80±3.03	0±0.92	-0.32±0.38	0.14±0.47	-0.17±0.80	1.17±0.55	0.01±0.87	0.45±0.96	0.51±0.67
14 day-old	-0.47±0.33	-0.43±0.17	0.46±0.37	-0.13±0.35	-0.53±0.33	0.15±0.21	-0.10±0.20	-0.70±0.60	0.07±0.23	-0.85±0.79
21 day-old	0.12±0.20	-0.12±0.11	0.03±0.20	0.05±0.12	0.58±0.26	0.03±0.15	0.47±0.28	0.78±0.38	0.88±0.50	1.28±0.53
42 day-old	0.33±0.42	-0.48±0.03	-0.30±0.14	-0.70±0.24	-0.75±0.27	-0.43±0.57	-0.33±0.44	-0.63±1.00	-0.40±0.25	-0.03±0.33

Note : Cell entries are mean differences between pre- and post-test temperatures (°C) ± one S.E.M. There were no differences found in pre and post-test temperatures between the different chronic treatment groups. N = 3 - 7 per cell.

Discussion

These data demonstrate that naltrexone injected 7, 14, 21, and 42-day old rats that had been chronically treated with morphine show an overall pattern of opiate withdrawal characterized by different behaviors at each age. For example, head moves, moving paws, rolling, and stretching occurred predominantly in the preweaning animals. Behaviors such as burrowing, diarrhea, jumps, teeth chatter, and wet dog shakes occurred in the postweaning animals. Walking and quiet were developmentally continuous withdrawal behaviors occurring at all ages. Pups chronically treated with saline or left untreated did not show these behavioral changes. The data therefore indicate that morphine abstinent rats demonstrate withdrawal behaviors within the developmental repertoire of the animal.

The adult rat has been the focus of much attention for most investigators studying the opiate withdrawal syndrome. No single behavior or physiological change alone defines the morphine withdrawal syndrome in the adult rat, but rather withdrawal is characterized by a constellation of behavioral and physiological changes. Likewise the opiate withdrawal syndrome in the infant rat is characterized by a constellation of behavioral changes, albeit an age specific cluster of change. Behavioral and physiological signs of opiate withdrawal in the adult

include weight loss, decreased consumption of food and water, diarrhea, eye twitching, rhinorrhea, lacrimation, penile erection/ejaculation, increased sensitivity to touch, teeth chattering, increased exploring, jumping, and wet dog shakes. As the present study shows, naltrexone injected infant animals that had been chronically treated with morphine show increased rolling, stretching, head moves, and paw movements.

Quantification of specific behavioral changes unique to precipitated withdrawal in the infant animal is a necessary step to understand further the mechanisms of morphine dependence in the infant rat. In the adult rat, the assessment of physical dependence is based on the severity of the withdrawal and can be quantified by the intensity or frequency of a variety of vegetative and motoric withdrawal signs. Some signs become progressively more pronounced when dependence becomes stronger defined by increased doses of the opiate antagonist or higher doses of morphine, but not all increase signs concomitantly with increasing dependence. Writhing and wet dog shakes dominated when the dose was increased only to medium degrees; they decreased or disappeared when the dose was increased. This evidence indicates the existence of "dominant" signs that appear to suppress the "recessive" signs when withdrawal becomes stronger (Blasig et al., 1973). In the present study we observed that paw movement and

stretching were increased in the 7 day-old low morphine group (3 mg/kg). These behaviors decreased when the morphine dose increased (10 mg/kg). Walking became more dominant at the higher morphine dose. Furthermore, some behaviors showed linear dose-response curves for naltrexone (e.g. moving paws and quiet) whereas other behaviors showed inverse U-shaped dose response relationships (e.g. rolling and stretching). There is also evidence that suggests that when adult rats are chronically treated with morphine, different symptoms of withdrawal may be preeminent when the morphine antagonist is administered at different times after the last administration of morphine. Linesman (1977) found that locomotor activity, jumping and writhing are precipitated most frequently at shorter intervals after the last morphine injection; teeth chattering, wet shakes, ptosis, diarrhea, penile ejaculation, and hypothermia, are seen at longer intervals. Whether this is true of the infant rat is unknown.

The absence of these adult-like behaviors in the rat pup has led to the assumption that the withdrawal phenomenon does not occur at this age. This is likely due to the fact that the infant nervous system has not yet fully developed and therefore the motoric or autonomic systems that mediate these adult-like behaviors may not be apparent in the infant animal (Faneslow & Cramer, 1988). This alone should not lead one to assume that the rat pup does not experience morphine

withdrawal. Rather, it indicates that morphine abstinent rat pups demonstrate withdrawal behaviors that are appropriate to this age.

In the adult rat there is also a strong aversive component to opiate abstinence. Adult rats suppress drinking fluids and learn taste aversions (Glick, & Charap, 1973; Jackler et al., 1979; Schaefer & Michael, 1983), avoid entering places (Mucha et al., 1982; Mucha, 1987), and bury small objects to which they have been exposed during precipitated withdrawal (Mucha, 1991). The results of this study demonstrate only a series of overt behaviors that may constitute a withdrawal syndrome in the infant. Whether an aversive state also occurs in the infant rat is not known. It has been shown that there are increased levels of ultrasonic vocalizations following spontaneous or precipitated withdrawal that are suggestive of withdrawal in both infant and adult rats (Barr & Wang, 1992; Vivian & Miczek, 1991). Although ultrasonic vocalization in the rat pup may serve as a signal to the dam that the pup has become separated from the litter (Allin & Banks, 1972; Noirot, 1972; Smotherman et al., 1974), there is also evidence that suggests that these vocalizations may serve to indicate some internal "dysphoric" state. Several groups have demonstrated that stressful stimuli such as isolation, cold ambient temperature, and frustrative non-reward elicit crying in the rat pup (Amsel et al., 1977; Bell, 1971; Hofer & Shair, 1987). Thus ultrasounds may be a

behavioral sign of opiate withdrawal or may represent the affective component of withdrawal.

It has been suggested previously that drug withdrawal *in utero* may be responsible for the adverse affects of maternal drug addiction observed in the infant (Lichtblau & Sparber, 1981; Kuwahara & Sparber, 1981). In order to precipitate withdrawal *in utero*, morphine dependent rats were injected with naloxone during gestation (Lichtblau & Sparber, 1981). Treatment with naloxone in this manner increased the number of stillbirths, decreased pup weight and size, and induced weight loss 24 hours after birth. In an effort to study the direct effects of opiates on the fetus with the absence of maternal-fetal interaction, Kuwahara & Sparber (1981) injected dependent fetal chicks with naloxone and found the motility of the opiate-exposed fetuses was significantly increased and hatchability significantly reduced compared to control fetuses. After continuous morphine infusions into pregnant ewes, naloxone injected into their fetuses produced increased fetal arterial blood pressure, decreased fetal heart rate, arterial blood pH and PO_2, and defecation of meconium (Cohen et al., 1980). When the pregnant ewes were injected with naloxone, the same responses were observed in the fetuses and opiate withdrawal was induced in the mothers.

These data taken together indicate that both the fetus and infant do

experience withdrawal from opiates and abstinence may be in part responsible for some of the adverse effects of opiates observed in animals as well as humans. The results of the present study clearly show that a morphine withdrawal-like syndrome can be described in the rat pup. Precipitated withdrawal at 7 days of age increased behaviors within the developmental repertoire of the pup, including rolling, stretching, and paw movements. They did not, however, show wet dog shakes, jumping, and teeth chatter that are characteristic of adult withdrawal. Behaviors such as these that are more typical of adult-like withdrawal did not occur until the animal was at least 21 days old. Therefore, the pup can demonstrate withdrawal-like behaviors that are different from those of the adult. Classification of these behaviors provides a necessary stepping stone to the understanding of the mechanisms that underlie the morphine withdrawal syndrome in the infant.

Chapter 3

Opiate Withdrawal in the Fetal Rat: A Behavioral Profile

Introduction

Exposure to opiate drugs such as heroin and morphine during pregnancy has been associated with a variety of adverse effects that range from fetal and neonatal death to premature birth, chromosomal aberrations, and decreased birth weights and head circumferences (Besunder & Blumer, 1990). Because opiates readily cross the placental barrier, these deleterious effects may be in part a result of the placental transport of the drug. Since one common characteristic observed in the pattern of drug use among pregnant women is that of repeated exposure and withdrawal, some of these effects may in fact be a result of the fetus experiencing withdrawal from the drug *in utero*.

The most marked and persistent neonatal complication associated with maternal opiate exposure is the period of withdrawal that begins within 1 - 3 days of birth (Hans, et al., 1984). In the human infant, the most common withdrawal symptoms include respiratory and gastrointestinal dysfunction, yawning, sneezing, and fever, frantic sucking of fists, an inconsolable high pitched cry, restlessness and irritability, and difficulty with feeding (Finnegan, 1976). Unfortunately, interpreting this data in humans is difficult because the drug-dependent woman is predisposed to a whole host of maternal complications. For example, a majority of the opiate addicts who become pregnant are of a low socio-economic status and

have a history of multiple drug abuse often paralleled with mental or medical illness (Hutchings, 1990). Many of these women get inadequate prenatal care, and are undernourished.

In order to examine the effects of prenatal opiate exposure in the absence of maternal complications we must turn to the animal model. In the adult rat, withdrawal of opiates or administration of opiate antagonists in opiate-exposed animals precipitated a withdrawal syndrome. This syndrome is characterized by a variety of vegetative and motoric signs including weight loss, decreased consumption of food and water, diarrhea, eye twitching, rhinnorrhea, lacrimation, penile erection/ejaculation, increased sensitivity to touch, hostility on handling, teeth chattering, increased exploring, jumping, and wet dog shakes. Although the withdrawal syndrome in the adult animal is well defined, the available data describing the withdrawal syndrome in the developing animal are limited. In the rat, prenatal opiate exposure has effects parallel to those in the human, such as increased fetal and neonatal death and lower birth weights (Hans, et al., 1984; Lichtblau & Sparber, 1981; Smith, et al., 1973). Past studies examining the neonatal withdrawal syndrome in the rat report that although adult-like withdrawal does not occur until well after weaning in the rat (Fanselow & Cramer, 1988; Jones & Barr, 1995), a neonatal withdrawal syndrome characterized by increased

levels of ultrasonic vocalizations and specific behaviors appropriate to the age of the animal does occur (Barr & Wang, 1992; Jones & Barr, 1995; Windh, et al., 1995). Morphine-treated rat pups, as young as 7 days of age, tested with naltrexone show increased rolling, stretching, head and paw movements.

The existence of a fetal withdrawal syndrome in the rat has been suggested based on the effects of morphine and naltrexone on the spontaneous activity in rat fetuses. Kirby (1979, 1981a) found that morphine caused a depression of activity in fetuses from the 15th through 21st day of gestation that was reversed by naloxone. However, a detailed behavioral profile of opiate withdrawal in the fetal animal has not been described. In an effort to describe behaviors that may reflect opiate withdrawal *in utero*, we precipitated withdrawal in morphine-exposed GD 20 fetuses (Gestation Day 20), and measured the resultant changes in behavior.

Methods

Subjects. The subjects were fetuses from Long-Evans hooded rats mated in our colony. The parent rats were housed in plastic tubs in a colony room maintained at 22 - 24 °C on a 12-hr light-dark photocycle with light onset at 7 AM. Every morning, females were checked for the presence of sperm in the vaginal smears. Once sperm were detected, the male was separated from the female and that day was termed gestation day 0 (GD-0). All studies followed NIH

guidelines.

Methods for generating dependence

Treating the dam. Drugs were delivered to the fetus indirectly through the dam. On gestation day 14, the dam was anesthetized with Isoflorane and implanted with a 75.0 mg morphine pellet (generously supplied by NIDA), a standard technique used to produce opioid dependence in laboratory animals (Advokat, 1981; Cicero & Meyer, 1973; Yoburn, 1985). Controls included dams that underwent the implant procedure but received no pellet.

Methods for precipitating withdrawal

Under anesthesia (Isoflurane), each female received an injection of 100% ethanol (100 µl, delivered at room temperature) into the spinal cord between the first and second lumbar vertebrae (Smotherman & Robinson, 1986). Following the spinal injection, each female was completely unresponsive to stimulation. She was then gently restrained in a holding apparatus, the uterus was exteriorized through a midline laparotomy, and her uterus and hindquarters were immersed in a temperature-controlled (37.5°C) bath containing isotonic saline. The uterus was exteriorized through a midline laparotomy and the number of fetuses in each uterine horn were counted. The mother and fetuses were allowed to recover from the anesthesia and to acclimate to the water bath for 20 minutes before behavioral

observations began. Four subject fetuses were then selected for behavioral observation, two from each uterine horn. The first of the four subject fetuses was prepared for direct observation by the delivery of the subject fetus through a 10 - 15 mm incision in the uterine wall, maintaining the placental-uterine attachment intact, with removal of the amniotic membranes from around the fetus (Smotherman & Robinson, 1986). The average weights of the fetuses was determined in preliminary studies. The fetus was injected s.c. with naltrexone (approximately 1.0 mg/kg) and its behavior was recorded every 15 seconds for a total of 10 minutes using a behavioral checklist (Table 5). This checklist was developed by integrating *in utero* behaviors outlined in previous investigations describing spontaneous behaviors that occur in the fetal rat (Smotherman & Robinson, 1986). When that observation period ended, the fetus was anesthetized to quiet it and prevent interference with the next observed fetus.

Statistics. A factorial analysis of variance (ANOVA) was conducted for each behavior. The 10-minute observation period was divided into 2 different time periods, each consisting of 5 minutes. Behaviors were summed in each time period, which was treated as a within-subjects variable. Doses were injected

Table. 5. Behavioral Definitions of the Fetus

Body Curls	Ventral or lateral flexion of the trunk.
Body Twitch	Brief spasms along the flank of the fetus.
Face Wiping	Wiping one or both forelimbs across the face.
Foreleg Movements	Flexion, extension or rotation of one or both hindlegs.
Head Movements	Ventral, dorsal, or lateral rotary motion of head.
Hindleg Movements	Flexion or extension of one or both hindlegs.
Mouth Movements	Opening and closing the mouth.
Quiet	Sedated appearance with no movement.
Stretch	Extension or dorsal flexion of the trunk resulting in an apparent lengthening of the body.

within a single litter, and treated as a within-subjects variable. The different chronic treatment groups served as the between-subjects variable.

Results

The results demonstrate increased fetal activity precipitated by naltrexone in morphine exposed animals. No significant time effects were observed. Face wiping occurred in all morphine exposed animals treated with naltrexone but never occurred in any other treatment group. Therefore, no analysis was performed for this behavior. The following behaviors showed significant interaction effects between acute treatment and chronic treatment (Figure 5): Body curls, ($F_{(1,7)} = 23.51$, $p<.001$) Forelimb movements, ($F_{(1,7)} = 25.10$, $p<.001$); Hindlimb movements, ($F_{(1,7)} = 11.57$, $p<.01$); Mouth movements, ($F_{(1,7)} = 13.94$, $p<.01$); and Quiet, ($F_{(1,7)} = 30.08$, $p<.001$). Behaviors demonstrating no significant effects were body twitch, head movements, and stretch.

Figure 5. Mean Number of occurrences per 10 minute observation period of fetal behaviors that showed a significant interaction between acute treatment and chronic treatment. Chronic treatment groups included morphine exposed and saline treated animals. There were a total of 5 morphine treated dams, and a total of 6 saline treated dams. Four subject fetuses were tested per dam. Two subject fetuses were treated with saline, and two subject fetuses were treated with naltrexone.

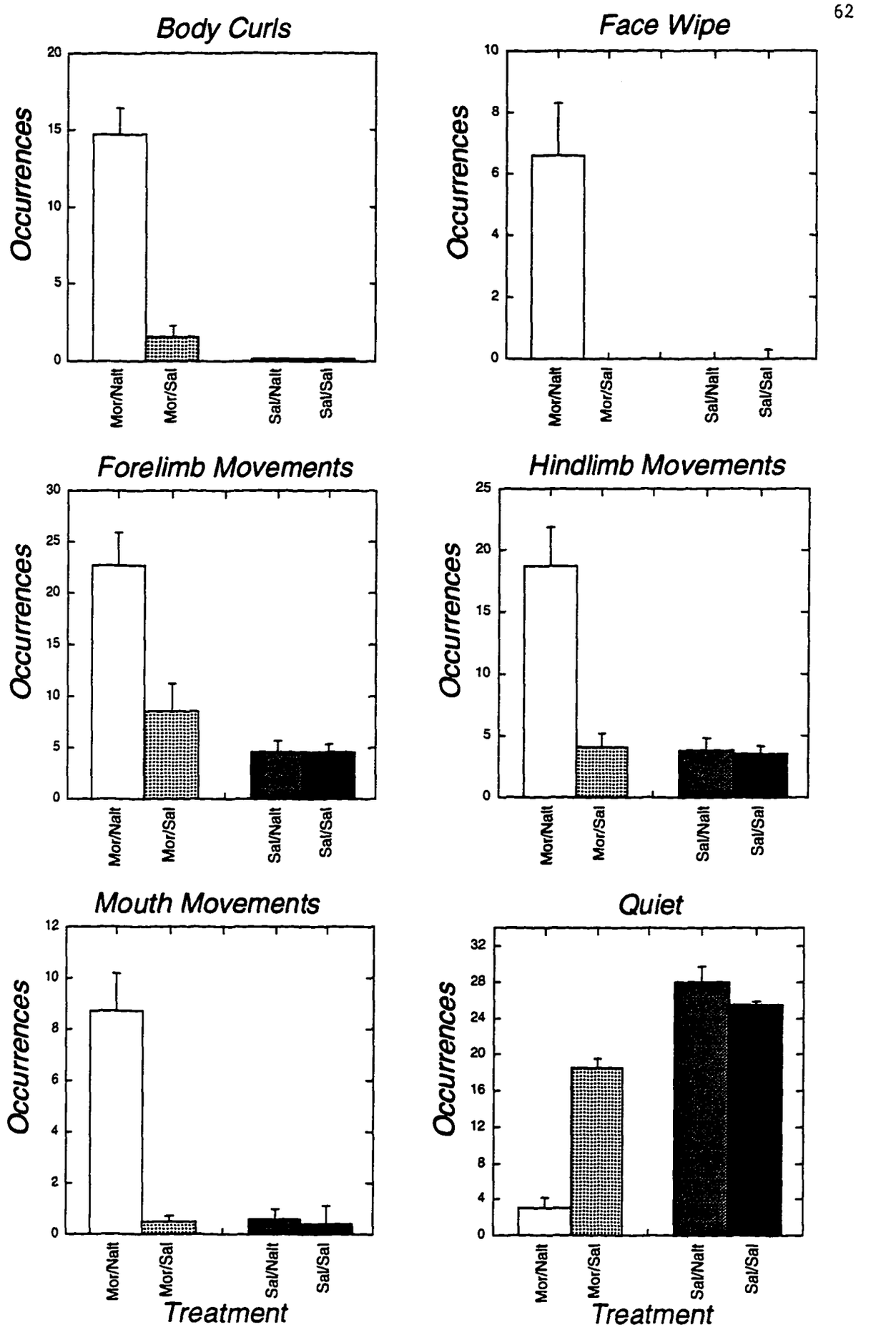

Figure 5

Figure 6. Mean number of occurrences per 10 minute observation period of fetal behaviors that demonstrated no significant effects. Chronic treatment groups included morphine exposed and saline treated animals. There were a total of 5 morphine treated dams and a total of 6 saline treated dams. A total of 4 subject fetuses were tested per dam. Two subject fetuses were treated with saline, and two subject fetuses were treated with naltrexone.

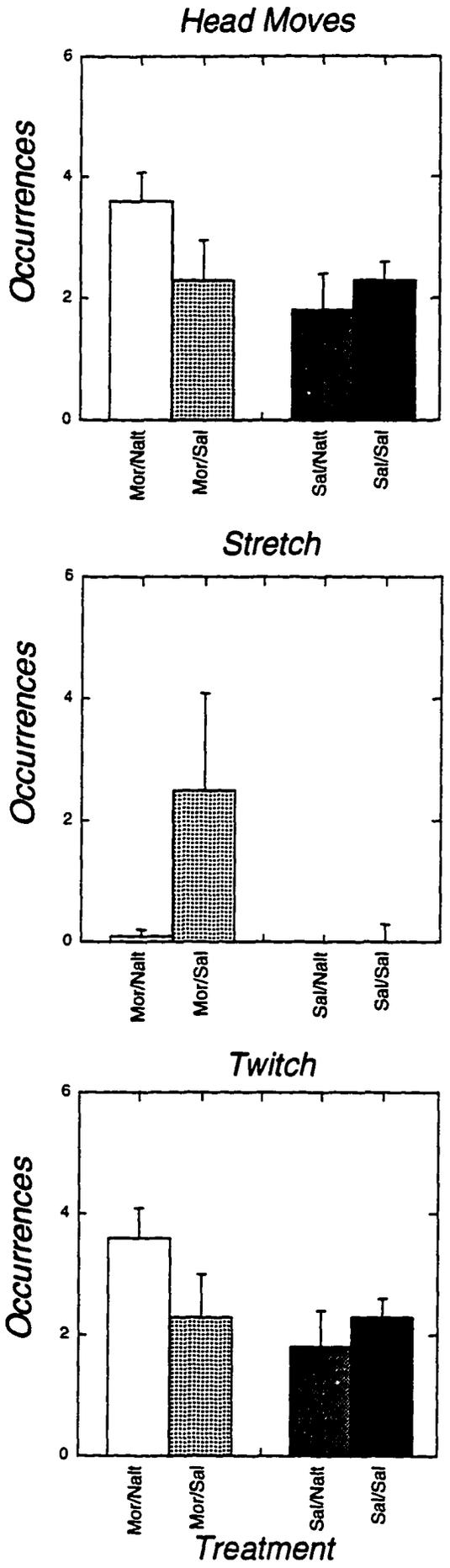

Figure 6

Discussion

Although the presence of a withdrawal syndrome in the fetal animal has been previously hypothesized (Kirby, 1979; Kirby, 1981a,b; Kuwahara & Sparber, 1981; Lichtblau & Sparber, 1981), this is the first study to provide a detailed behavioral profile that defines the prenatal opiate abstinence syndrome. The present study indicates that naltrexone-injected GD 20 fetuses that had been passively exposed to morphine (through the placental transport of the drug) show a specific alteration in behavior patterns consisting of increased body curls, limb and mouth movements, face wiping, and less quiet time as compared to control animals. Other behaviors such as body twitching, head movements, and stretching do not change.

Several animal models have been used to examine the effects of opiate withdrawal *in utero*. Lichtblau & Sparber (1981) found that litters from dams put through withdrawal daily with naloxone injections from day 14 of gestation through term, showed an increased rate of stillbirths, decreased pup weight and size as well as weight loss 24 hours after birth as compared to morphine dependent saline treated rats. When morphine-dependent fetal chicks were injected with naloxone, the motility of the opiate-exposed fetuses was significantly increased and

the hatchability significantly reduced as compared to control fetuses (Kuwahara & Sparber, 1981). Following an injection of naloxone into fetuses from morphine-exposed ewes, fetal arterial blood pressure and defecation increased, and fetal heart rate decreased (Cohen, et al., 1980). Using the chronically instrumented fetal lamb preparation, Szeto's group has characterized a syndrome of naloxone-precipitated opiate abstinence following short-term infusion of morphine into the fetus (Umans, & Szeto, 1983). The syndrome consisted of desynchronization of electocortical activity; increased total body movements, neck tone, and eye movements; continuous, rapid deep breathing movements; and immediate bradycardia associated with transient increases in systolic, diastolic, and pulse pressures; and meconium staining of the amniotic fluid (Umans, & Szeto, 1983). These data suggest that an intrauterine opiate withdrawal presents a severe physiologic stress to the developing fetus.

The effects of morphine on spontaneous activity in the rat fetus have also been observed. Fetal movements begin on the 15th day of gestation. Kirby (1979) injected the dam with morphine on gestation days 7 through 17 and observed that morphine decreased spontaneous activity on day 18. Spontaneous fetal activity from days 15 to 21 was decreased equally at all ages following a maternal injection of morphine and this decrease was reversed by naloxone.

In the present study, we delivered the subject fetus through a uterine incision, maintaining the placental-uterine attachment, and removed the amniotic membranes from around the fetus. This allowed us to inject the fetus subcutaneously with naltrexone and to directly observe the fetus. Observed fetal activity was then categorized with reference to the region of the body that was moved. We were able to identify the same basic patterns of fetal movement as previously reported by Smotherman & Robinson (1986) in their study of spontaneous behavior in the fetal rat. The results of the present study show that behaviors that spontaneously occur in the fetus, such as body curls, mouth and limb movements, were greatly increased in naltrexone injected fetuses chronically exposed to morphine as compared to control animals. Other spontaneous behaviors such as body twitch, head and mouth movements, and stretching did not increase significantly in experimental animals. Face wiping was unique in that it only occurred in morphine exposed animals treated with naltrexone. This behavior involved a synchronous movement of the two forelegs along the side of the head and closely resembled grooming patterns observed in adolescent and adult rats during withdrawal (Jones & Barr, 1995). Smotherman & Robinson (1992), characterize face wiping as a pattern of motor behavior rarely seen in the absence of aversive stimulation such as a tactile probe or chemosensory stimulation.

Smotherman & Robinson's (1992) findings taken together with the present results suggest that face wiping may be a motor response evoked by aversive stimuli.

Quantification of these specific behavioral changes associated with the fetal abstinence syndrome provides some insight into how the effects of opiates and opiate withdrawal on fetal behavior may contribute to the adverse effects observed in offspring of drug-dependent women. For example, past research has emphasized the placental transfer of opiates as the primary mechanism involved in the deleterious effects associated with maternal opiate dependence. Although this mechanism accounts for the direct effects of placental transport (Ricalde & Hammer, 1990), it ignores the complexity of the fetal environment, which is constantly interacting with the fetus. Motor activity in the fetus is necessary to promote normal behavioral as well as morphological development (Smotherman & Robinson, 1992). It has been previously suggested that these behaviors are not just trivial aspects of the existence of the fetus but rather they are ontogenetic adaptations designed to promote the survival and growth of the fetus (Oppenheim, 1982; Smotherman & Robinson, 1987). Therefore anything that influences the behavior of the fetus may be a potential threat to normal development. For example, periodic episodes of heroin withdrawal during pregnancy restrict fetal growth by reducing uterine or placental bloodflow (Naeye, 1965). Episodes of

maternal withdrawal will increase the activity of the fetus, thereby increasing the metabolic rate and oxygen consumption of the fetus. As a result, the fetus may experience hypoxia or even death (Finnegan, 1976). Fetal hypoxia is known to alter fetal cardiorespiratory and neurobehavioral function (Szeto, 1995). For example, acute hypoxia has been shown to cause fetal bradycardia and decrease rapid eye movement (REM) sleep and fetal breathing activity (Boddy et al., 1974; Koos et al., 1987). Thus the effects caused by opiate withdrawal may be a consequence of both direct and indirect effects of abstinence.

Much data exist on the opiate withdrawal syndrome in the adult rat and emerging data now indicate the existence of a distinct abstinence syndrome in the neonatal animal. For example, naltrexone-injected 7- to 42-day-old rat pups that had been chronically treated with morphine showed an overall pattern of opiate withdrawal characterized by different behaviors at each age. Behaviors such as head and paw movements, rolling, and stretching occurred predominately in the preweaning rats whereas behaviors such as burrowing, diarrhea, jumps, teeth chatter, and wet dog shakes that are more typical adult-like behaviors occurred in the postweaning rats. Walking and quiet were developmentally continuous withdrawal behaviors occurring at all ages. Rats chronically treated with saline or left untreated did not show these behavioral changes (Barr & Goodwin, 1997;

Fanselow & Cramer, 1988; Jones & Barr, 1995; Windh, et al., 1995). Ultrasonic vocalizations also increase following spontaneous or precipitated withdrawal in the infant rat (Barr & Wang, 1992; Zmitrovich, et al., 1993). These results clearly show that a morphine withdrawal-like syndrome can be described in the rat pup when behaviors are examined that are within the animals developmental capacity to perform.

Although recent data is consistent regarding the behavioral components of opiate withdrawal in the neonatal rat, little is known regarding cellular and molecular mechanisms in the developing animal. Investigations of these mechanisms in the adult rat now indicate that changes in the activity of G-proteins, cAMP second messengers, and protein phosphorylation in part mediate the aspects of opiate dependence and withdrawal (Nestler, 1992; Rubino, et al., 1995; Trujillo & Akil, 1991). Although some studies report changes in opiate receptor binding after perinatal opiate exposure (Tempel, et al, 1988; Tempel, 1991; Zadina, et al., 1985) others report no alterations in receptor affinity or number in neonates (Windh, et al., 1995). Since the neonatal animal experiences an opiate withdrawal syndrome that is characteristically distinct from that of the adult animal, there is no reason to assume that the underlying mechanisms are the same as that of the adult. For example, in the mature organism, the adrenal medulla acts in concert with the

sympathetic nervous system to coordinate cardiovascular and metabolic responses to stressors such as hypotension, hypoglycemia and hypoxia. In the fetus and neonate, however, sympathetic efferent neural pathways are generally immature or non-functional, and consequently the adrenal medulla plays a predominating role in mediating adrenergic responses (Slotkin & Seidler, 1988a). Slotkin's group has shown that opiate exposure on peripheral sympathetic neuronal development display selective actions involving increased impulse activity, accelerated synaptogenesis and direct, opiate-receptor-mediated suppression of non-neurogenic neonatal adrenomedullary secretory responses (Slotkin, 1988b). Accelerated neuronal competence in the periphery and interference with specialized adrenal catecholamine mechanisms contributes to the greater risk of mortality in the perinatal opiate syndrome, and may also have a long-term impact on cellular development of non-neural structures (Slotkin, 1988b).

The recent description of behaviors associated with opiate withdrawal in neonates as well as the present results indicating a fetal abstinence syndrome will provide the basis for a clearer understanding of the cellular and molecular mechanisms that mediate the opiate withdrawal syndrome in the developing animal.

Chapter 4

Methylnaloxonium Injection into the Locus Coeruleus and Periaqueductal Gray but not the Amygdala Precipitates Morphine Withdrawal in the 7 day-old Rat

Introduction

The chronic administration of opiate drugs induces a state of physical dependence in both humans and animals. The physical component of morphine dependence is characterized by specific behavioral and vegetative signs following the cessation of opiate administration (spontaneous withdrawal) or by an opiate antagonist (precipitated withdrawal). The adult rat has been the model most commonly used to investigate the anatomical substrates that mediate the diverse symptoms of the opiate withdrawal syndrome. Opiate drugs act principally on the central nervous system (CNS), and the anatomical sites involved in the opiate withdrawal syndrome are widely represented within the CNS. These sites have been identified with a variety of technologies, all of which support the hypothesis that no single brain structure is entirely responsible for the classic signs of opiate withdrawal in the adult rat. The earliest studies involved the use of lesions. Lesions of the medial forebrain bundle, or of the ventromedial nuclei of the hypothalamus that interrupt this bundle, reduced several signs of opiate withdrawal (Glick & Charap, 1973; Kerr & Pozuelo, 1971). Thalamic, limbic, and cortical lesions did not alter the withdrawal syndrome (Adler, et al., 1978). Lesion of the central amygdala blocked the jumping behavior of the dependent animal without affecting the remaining signs (Calvino, 1979). Studies involving intracerebral

injections of opiate antagonists into specific brain structures in the adult rat also support the hypothesis of a diffuse distribution of multiple opiate withdrawal sites within the CNS. Areas found to be highly sensitive to the opiate antagonist naloxone are the medial hypothalamus, the periaqueductal gray (PAG)/4th ventricle area, amygdala, medial thalamus, and globus pallidus (Tremblay & Charton, 1981; Wei, et al., 1974). Because naloxone is a lipophilic opiate antagonist, these studies may have involved a rapid diffusion of the drug. Therefore, recent studies have utilized methylnaloxonium, is a hydrophilic opiate antagonist, in order to reduce the rapid diffusion of the drug. Methylnaloxonium binds competively to opiate receptor sites,is ten times less potent than naloxone (Koob, Randolph, and Chavkin, unpublished report); labeled methylnaloxonium remains at the site of injection much longer than labeled naloxone (Schroeder et al., 1991). The LC has been shown to be the most sensitive site for methylnaloxonium-precipitated withdrawal and methylnaloxonium injections elicited jumping, rearing, and locomotor activity. Injections of methylnaloxonium into the PAG also increased behaviors of rearing and locomotor activity. The least sensitive structures to methylnaloxonium-precipitated withdrawal were the amygdala, nucleus accumbens, and medial thalamus. Chronic administration of opiates into the brain show that anatomically distinct opiate receptor fields mediate

different components of addiction such as reward and physical dependence. For example, rats never before exposed to opiates rapidly learn to press a lever for microinjection of morphine into the ventral tegmental area. Challenge by a narcotic antagonist produced no signs of physical dependence. Dependence was not seen after long-term morphine infusions into the ventral tegmentum but was seen after similar infusions into the PAG (Bozarth & Wise, 1984).

Collectively, these studies have mapped out the anatomical substrates involved in the opiate withdrawal syndrome. Although this neural map is complex and multi-faceted, it has provided a model to further investigate the adaptive processes associated with the opiate withdrawal syndrome in the adult rat at the cellular and molecular level. Whereas much progress has been made in our understanding of how the behavioral, anatomical and molecular components function together to produce the opiate withdrawal syndrome in the adult rat, virtually nothing is known regarding the interactions of these mechanisms in the developing animal. This lack of information is due in part to the fact that the behavioral signs associated with this syndrome in the developing animal are distinct and different from the classic signs of opiate withdrawal observed in the adult animal. Until very recently there has been no detailed behavioral description that profiles the opiate withdrawal syndrome in the developing animal. Two labs have

now completed extensive investigations describing the maturation of the opiate withdrawal syndrome as it is manifested in the fetal, neonatal, and adolescent animal. At each level of development, precipitated withdrawal was evidenced by a distinct and consistent behavioral syndrome, but the nature of that syndrome changed with age. The classical withdrawal signs associated with adult opiate withdrawal did not appear until puberty (Jones & Barr, 1995; Windh, et al., 1995). These data together provide a model detailing the physical component of withdrawal at each different stage of development and thereby setting the stage for the study of the anatomical substrates, as well as cellular and molecular mechanisms, that mediate the opiate withdrawal syndrome in the developing animal. The goal of this study is therefore to determine if the changing face of opiate withdrawal during development is mediated by similar or different neural sites as in the adult. We therefore precipitated opiate withdrawal in morphine dependent 7 day-old rat pups by injecting methylnaloxonium into one of three different brain structures, the amygdala, LC, or the PAG and measuring the resultant changes in behavior.

Methods

Subjects. The subjects were offspring of Long-Evans hooded rats mated in our laboratory. The parent rats were housed in plastic tubs in a colony room

maintained at 22-24° C with a 12-hr light-dark photocycle with light onset at 7 a.m. Cages were checked twice daily at approximately 10 a.m. and 6 p.m. Pups found at either time were termed 0 days of age. After parturition, litters were culled to 8 pups without regard for the ratio of males to females.

Induction of morphine dependence. Animals were injected with morphine sulfate (10mg/kg ip) twice daily (10a.m. and 6 p.m.). From the first to the 7th day of life. Controls included saline-injected pups. The last injection was on the morning of the 7th day. The order of the treatment conditions were assigned randomly with the exception that a maximum of six litters were used per condition.

Cannula Implantation. On the evening of the sixth day the animals were transported from the animal facility to our labs in plastic tubs with bedding and placed in an observation chamber and maintained at approximately 33° C. One rat was then fully anesthetized with Metafane and placed in a stereotaxic device adapted for infant animals. The field was prepped and the skin and muscle layers reflected. A burr hole was made in the skull, and the cannula placed to the required depth, and anchored with dental cement. The incision was closed with cyanocrylate cement. A total of 8 pups per litter underwent surgery. Cannula guides were permanently kept open with wire stylets which were removed for intraventricular injections. Following surgery pups were kept in a warm, moist

incubator (34° C) overnight. Prior to behavioral testing on day 7, pups were fed milk (half and half) from a plastic pipette to help maintain hydration. These methods are in routine in our lab and have been described previously (Barr, 1991). The coordinates for amygdala placements were A.P., -2.0, M.L., -3.6, and D.V. -2.4. Locus coeruleus coordinates were A.P., -2.8, M.L., -.8, and D.V., -1.7. PAG coordinates were A.P., -4.2, M.L., -.5, and D.V. -2.3.

Induction of morphine withdrawal. On the afternoon of the 7th day, one pup was weighed, rectal temperature recorded, and injected with saline or methylnaloxonium (generously supplied by Boehringer-Ingelheim, Elmsford, NY) through the cannula. The following doses were injected in order to precipitate withdrawal: 30, 100, 300, 1000 ng/injection. A solution of 200 ml was injected over 70 s through a injector cannula (Plastic Products, 315I, 33 ga. 200mm) and associated tubing inserted into the guide cannula. The injector extended 1mm beyond the ventral edge of the guide. The external end of the tubing was connected to a 5 ul syringe (Hamilton) that was filled with distilled water. A small air bubble was left between the drug and water to prevent dilution and to serve as a calibration for the injection volume. After injection the cannula was left in place for an additional minute before being removed.

Behavioral Testing. Immediately following the injection, the pup was placed in an

observation chamber with the remainder of the litter. The pup was then observed for a period of 20 minutes and behaviors recorded every 15 s on a checklist. The checklist was developed in previous experiments by observing and recording behaviors displayed by morphine-treated pups after injection of naltrexone (Jones & Barr, 1995). When the observation period ended, rectal temperature was re-recorded and the pup was anesthetized and placed back into the litter. This was done in order to quiet the pup and prevent interference with the next observational pup. The second pup was then injected and tested in this manner.

Histological Verification. After completion of withdrawal testing, all the animals were deeply anesthetized with sodium pentobarbital and perfused with 10% formalin. After decapitation, the brains were removed from the skull, and cut on a cryostat at -20 C, and stained with cresyl violet. Representative sections showing the site of injection were then compared to standard stereotaxic plates (Sherwood & Timiras, 1970; Paxinos & Watson, 1986).

Statistics

A factorial analysis of variance (ANOVA) was conducted for each behavior in each different brain site. Since chewing and vocalizations were not detected during precipitated withdrawal in the PAG, analyses were not performed for these behaviors in the PAG. The 20 minute observation period was divided

into 5 different time periods, each consisting of 4 minutes. Behaviors were summed in each time period, which was treated as a within-subjects variable. All doses were injected within a single litter, and the drug dose effect was treated as a within-subjects variable. The different chronic treatment groups served as between-subjects variables.

Results

The neuroanatomical distribution of injection sites is shown in figure 8, 9, 11, 12, 13, & 14. In general, sites fell within a 1- to 2-mm cluster and are represented in drawings based on the atlas from (Paxinos & Watson, 1986; Sherwood & Timiras, 1970).

Microinjection of naltrexone in morphine dependent animals produced clear signs of withdrawal following injection of the PAG or LC, but not the amygdala. There were a total of two possible effects: chronic treatment effects (morphine vs. Saline-treated groups); and interaction effects between naltrexone dose and chronic treatment (see Table 6).

Locus Coeruleus. The administration of methylnaloxonium into the locus coeruleus of morphine dependent animals produced a severe withdrawal syndrome and significantly elicited the following signs of withdrawal: head movements, chewing, hindpaw movements, vocalizations, wall climbing, and spent less time

quiet as compared to control animals. Other signs such as rolling, stretching, and walking which are normally observed in the neonate following i.c.v. injections were not significantly observed. Animals receiving the highest dose of methylnaloxonium (1000 ng) engaged in continuous chewing movements and displayed a loud and clearly audible vocalization throughout the observation period. Animals with the final site of injection outside but in the proximity of the LC exhibited significantly less withdrawal.

Periaqueductal gray matter. The following signs were significantly elicited following administration of methylnaloxonium into the ventral PAG of morphine dependent animals: head movements, hindpaw movements, rolling, walking, wall climbing and spent less time quiet as compared to control animals. Paw movement and rolling were the most sensitive signs, showing a significant presence after administration of the lowest dose of methylnaloxonium (30ng).

Amygdala. Methylnaloxonium injections into the amygdala failed to elicit any of the physical signs of withdrawal in morphine dependent rat pups. There were no observable behavioral differences between the two treatment groups.

Table 6. Results of Analyses of Variance for Withdrawal Behaviors

Behavior	ANOVA (Dose X Chronic TX)	
	F	P
Chewing		
PAG	ND	
LC	(4, 36)=3.54	<.01
Amygdala	(4,40)=1.0	N.S.
Head Moves		
PAG	(4, 44)=2.36	<.06*
LC	(4,36)=7.55	<.001
Amygdala	(4,40)=1.98	N.S.
Paw Moves		
PAG	(4,44)=5.30	<.001
LC	(4,36)=11.47	<.0001
Amygdala	(4,40)=1.97	N.S.
Quiet		
PAG	(4,44)=21.26	<.0001
LC	(4,36)=48.62	<.0001
Amygdala	(4,40)=1.08	N.S.
Rolling		
PAG	(4,44)=5.63	<.001
LC	(4,36)=48.62	N.S.
Amygdala	(4,40)=1.13	N.S.
Separation		
PAG	(4,44)=1.45	N.S.
LC	(4,36)=0.44	N.S.
Amygdala	(4,36)=0.60	N.S.
Together		
PAG	(4,44)=0.89	N.S.
LC	(4,36)=0.84	N.S.
Amygdala	(4,40)=0.51	N.S.
Vocalizations		
PAG	ND	
LC	(4,36)=6.03	<.001
Amygdala	(4,40)=1.0	N.S.
Walking		
PAG	(4,44)=2.64	<.05
LC	(4,36)=1.81	N.S.
Amygdala	(4,40)=1.44	N.S.
Wall Climbing		
PAG	(4,44)=3.57	<.01
LC	(4,36)=2.75	<.05
Amygdala	(4,40)=1.36	N.S.

Note. ND=Not Detected.*Trend only.

Figure 7. This figure depicts the mean number (± one SEM) of occurrences of significantly elicited behaviors occurring following injection of methylnaloxonium into the LC. Chronic treatment groups included morphine exposed and saline treated animals. Chronic treatment groups included morphine (10 mg/kg) and saline injected litters. There were a total of 5 morphine treated litters, and a total of 6 saline treated litters. A total of 5 pups were tested per litter.

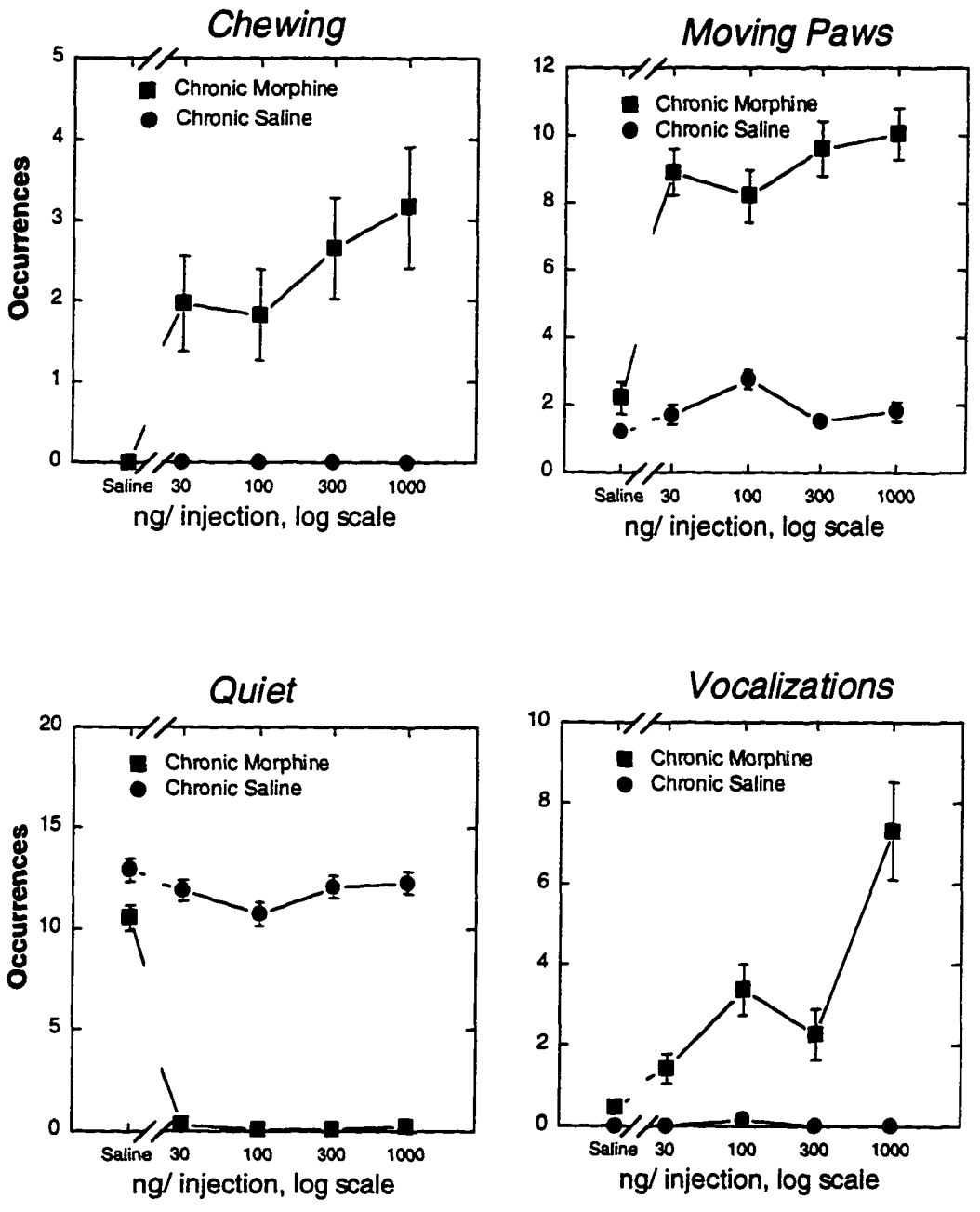

Figure 7

Figure 8. This figure depicts representative coronal sections of morphine-dependent rats showing the total number of occurrences for the behavior "chewing" at each injection site following injection of methylnaloxonium into the LC. This figure represents a total of 17 animals.

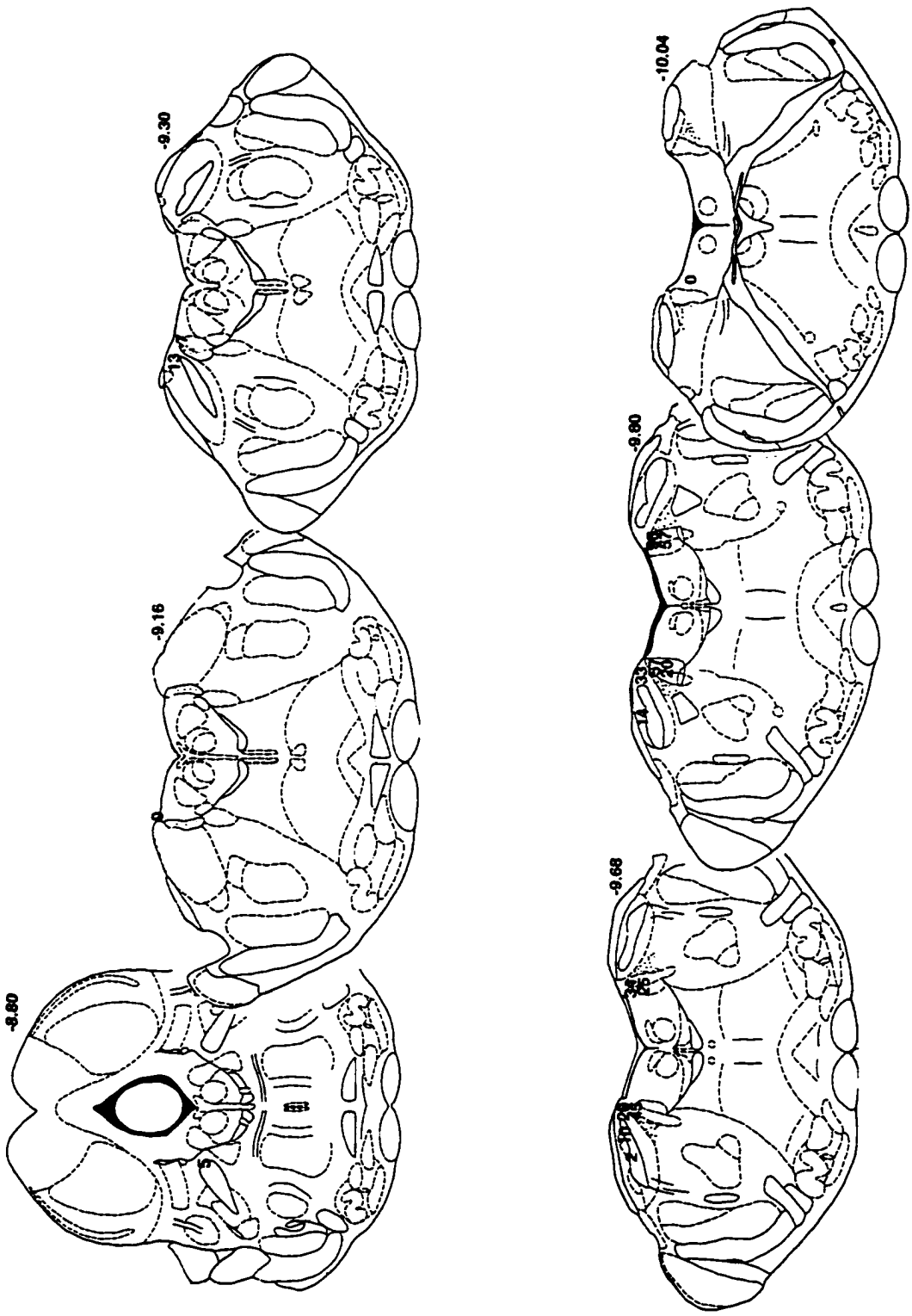

Figure 8

Figure 9. This figure depicts representative coronal sections of morphine dependent rats showing the total number of occurrences for the behavior "vocalizations" at each injection site following methylnaloxonium into the LC. This figure represents a total of 17 animals.

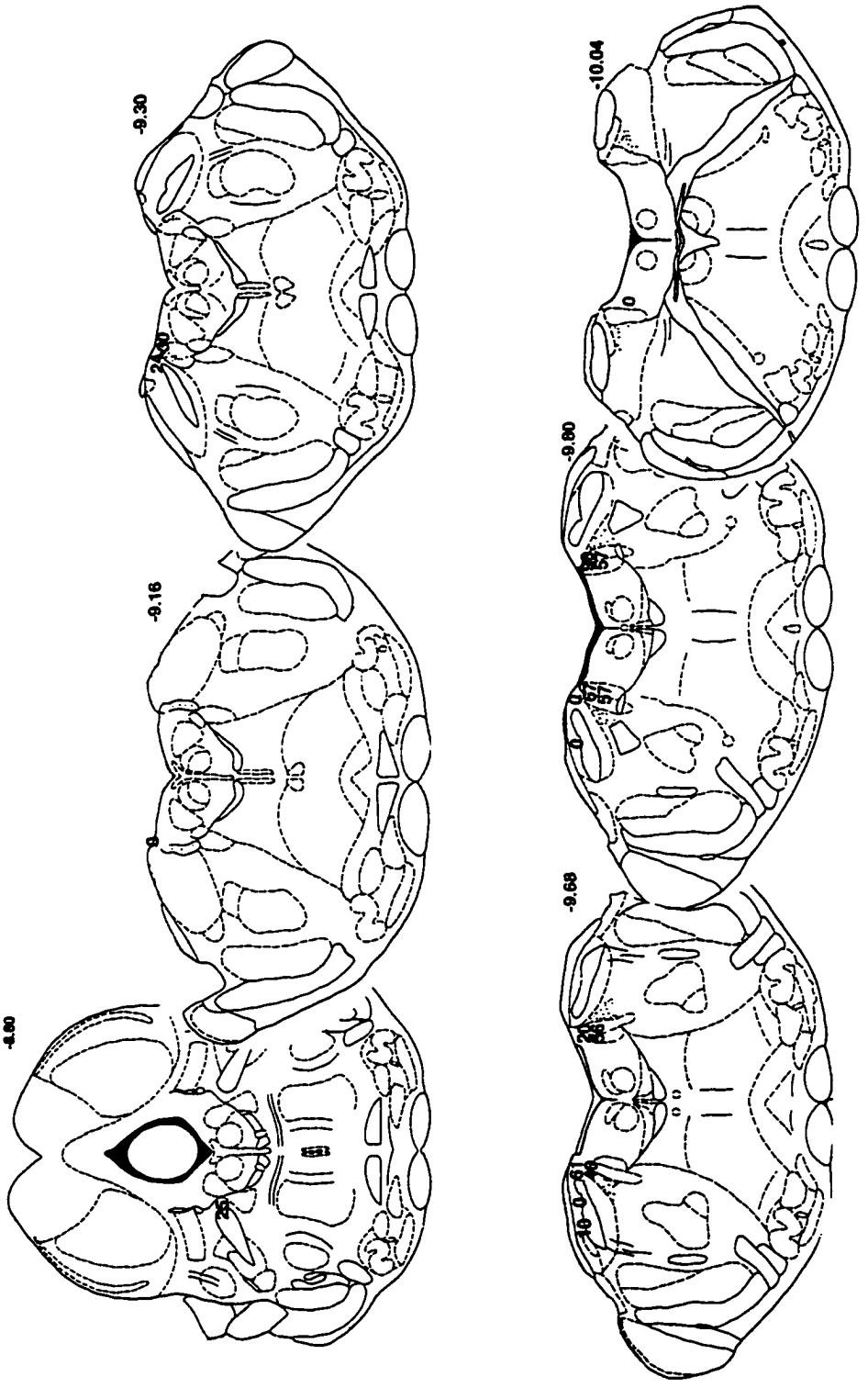

Figure 9

Figure 10. This figure depicts the mean number (± one SEM) of occurrences for behaviors significantly elicited following methylnaloxonium injection into the PAG. Chronic treatment groups included morphine exposed and saline treated animals. Chronic treatment groups included morphine (10 mg/kg) and saline injected litters. There were a total of 6 morphine treated litters, and a total of 6 saline treated litters. A total of 5 pups were tested per litter.

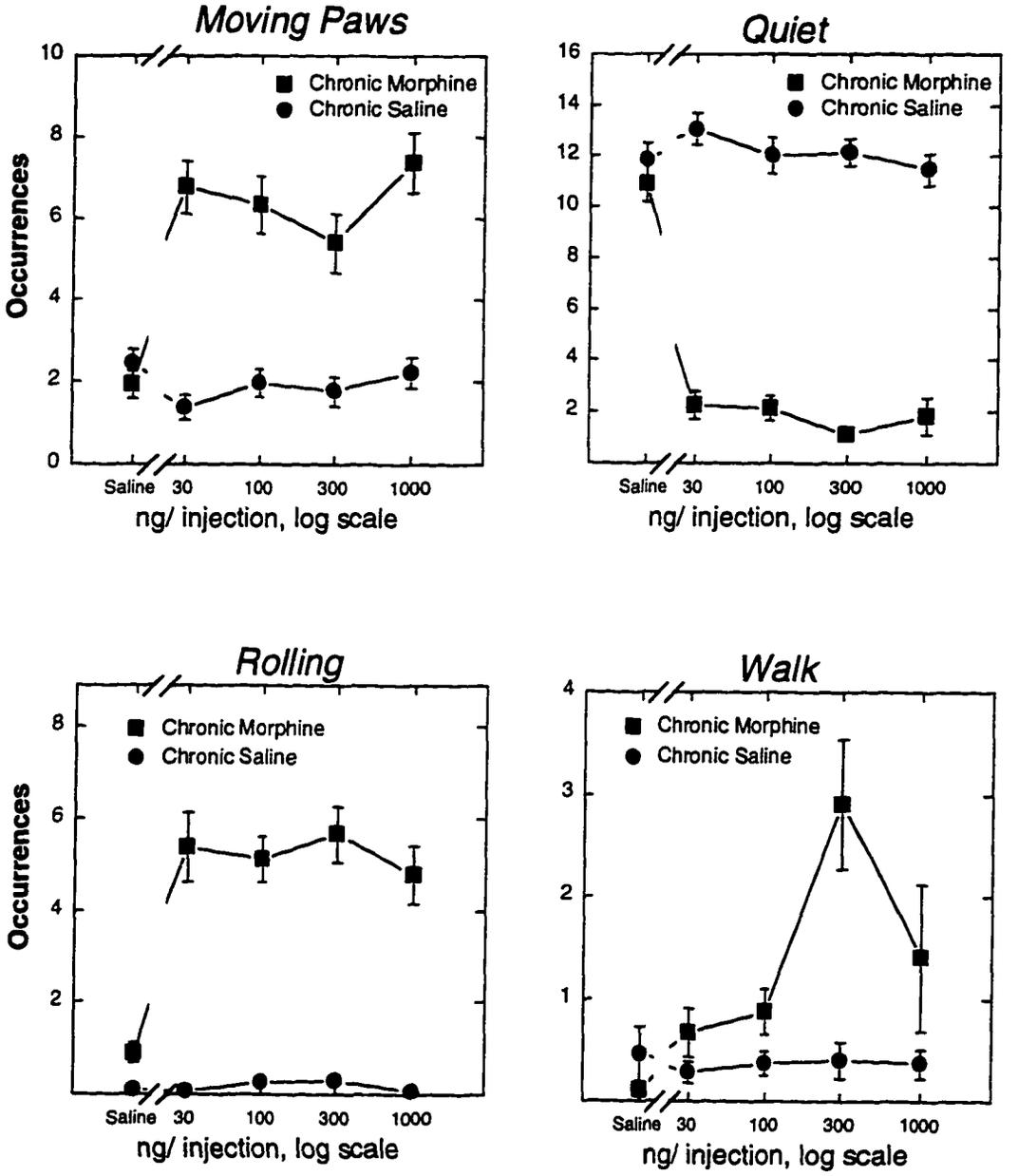

Figure 10

Figure 11. This figure depicts representative coronal sections of morphine dependent animals showing the total number of occurrences of the behavior "moving paws" at each injection site following methylnaloxonium injection into the PAG. This figure represents a total of 21 animals.

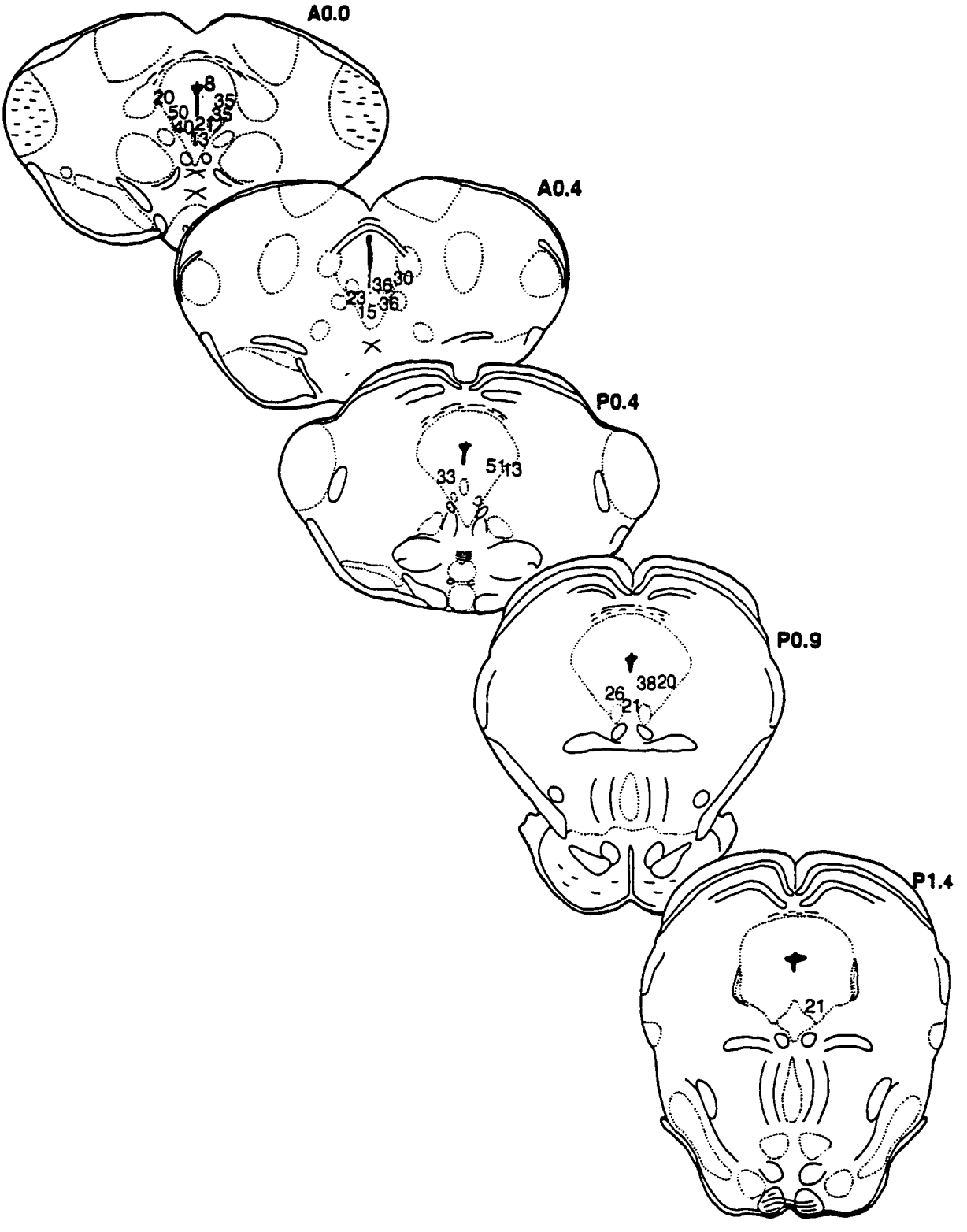

Figure 11

Figure 12. This figure depicts representative coronal sections of morphine dependent animals showing the total number of occurrences for the behavior "rolling" at each injection site following methylnaloxonium injections into the PAG. This figure represents a total of 21 animals.

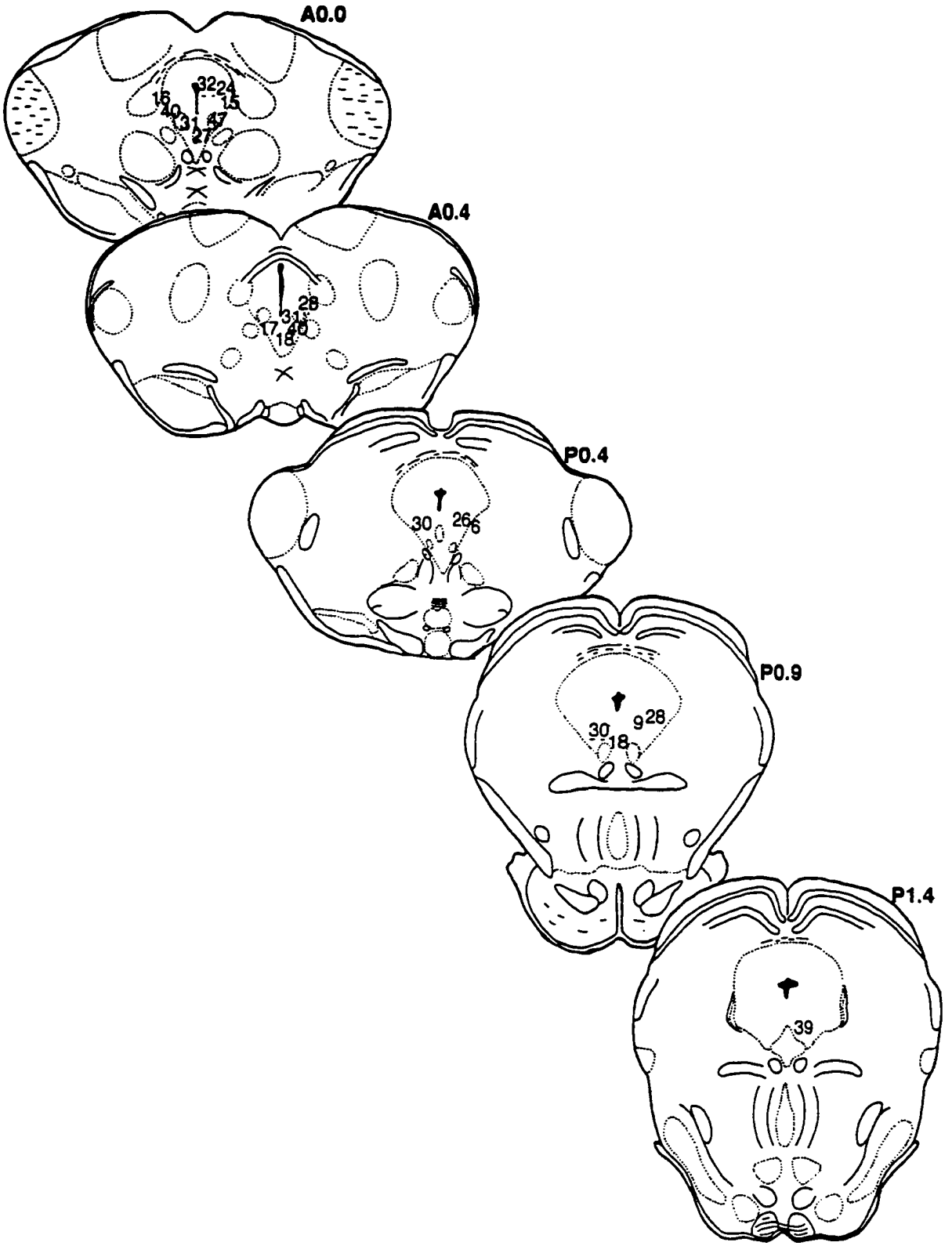

Figure 12

Figure 13. This figure depicts representative coronal sections of morphine dependent animals following injection of methylnaloxonium into the amygdala. This figure represents a total of 24 animals.

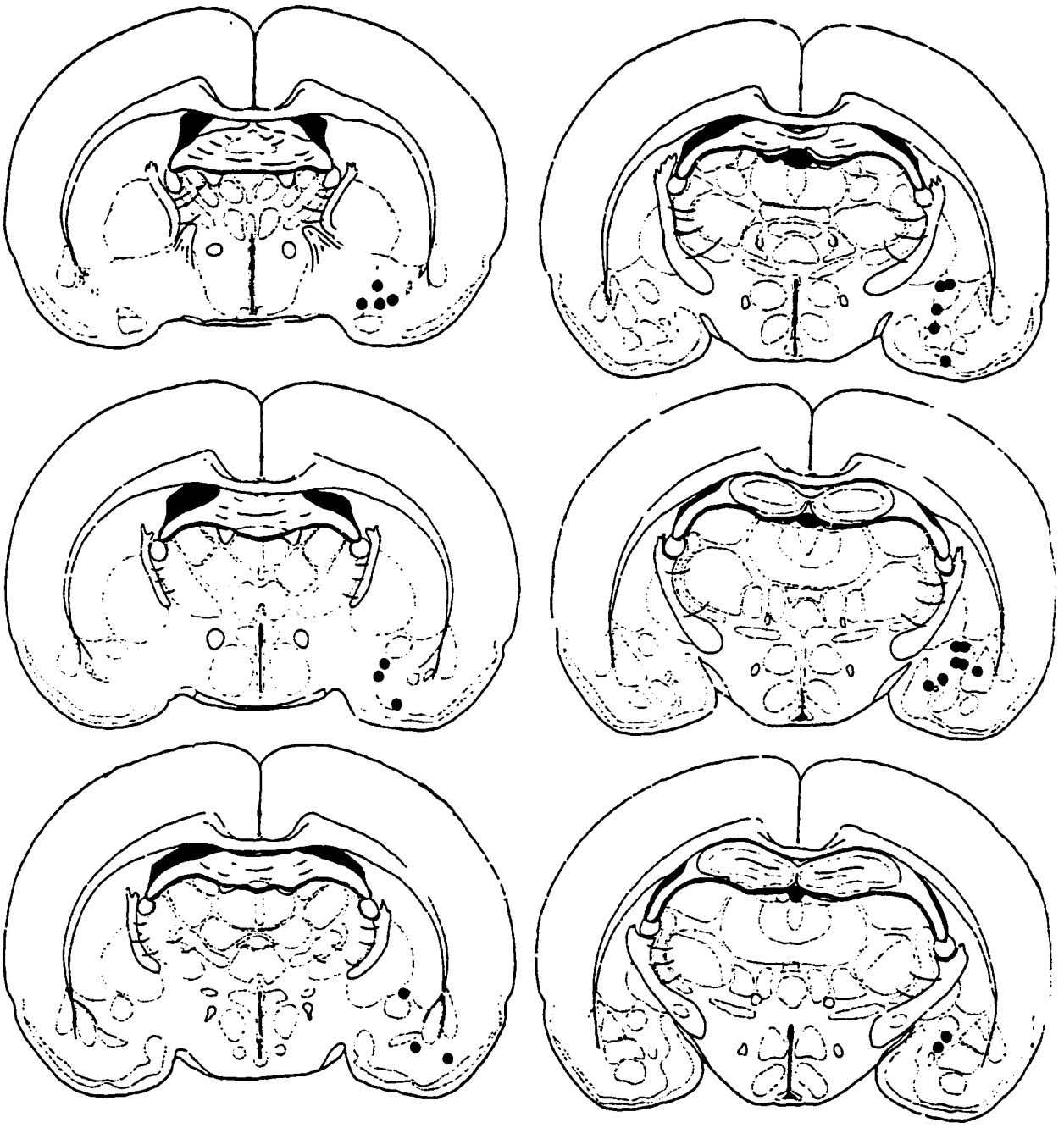

Figure 13

Figure 14. This figure depicts representative coronal sections of saline dependent animals following injection of methylnaloxonium into the amygdala. This figure represents a total of 24 animals.

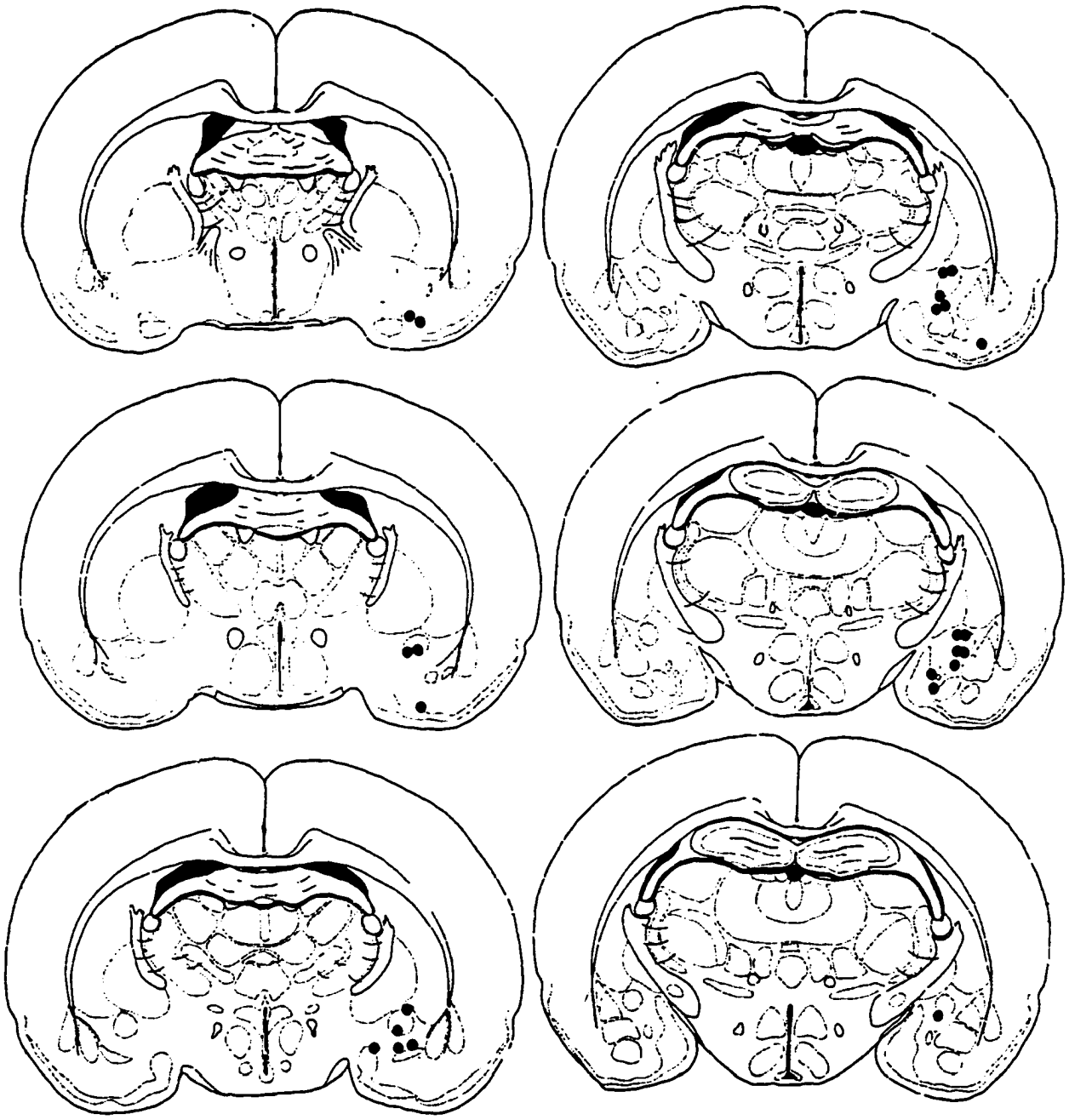

Figure 14

Table 7. Behaviors demonstrating no significant effects per brain site

Dose of Methylnaloxonium (ng/injection)

Amygdala	0	30	100	300	1000
Chewing	0.0	0.0	0.03±0.03	0.0	0.0
Paw Moves	0.50±0.14	2.30±0.38	1.86±0.32	1.53±0.32	1.57±0.28
Quiet	12.0±0.57	10.50±0.64	11.63±0.65	11.03±0.72	11.80±0.71
Rolling	0.0	0.03±0.03	0.0	0.0	0.03±0.03
Separated	1.37±0.72	0.07±0.07	0.33±0.30	2.37±0.95	2.57±1.0
Stretch	0.0	0.0	0.0	0.0	0.0
Together	12.53±0.90	14.37±0.53	14.0±0.62	11.77±1.05	11.57±1.0
Vocalizing	0.0	0.0	0.0	0.0	0.0
Wall Climb	0.0	0.03±0.03	0.0	0.13±0.10	0.03±0.03
Walk	0.07±0.05	0.73±0.20	0.30±0.14	0.77±0.28	0.50±0.20
PAG					
Separated	2.03±0.81	4.50±0.92	2.1±0.53	5.1±1.06	2.03±0.58
Stretch	0.0	0.0	0.13±0.13	0.0	0.0
Together	11.96±1.12	10.27±1.0	12.46±0.77	9.33±1.10	10.13±1.17
LC					
Rolling	0.0	0.55±0.23	0.20±0.12	0.55±0.20	0.60±0.15
Separated	1.4±0.61	1.68±0.44	2.32±0.72	1.84±0.52	1.68±0.58
Stretch	0.0	0.0	0.0	0.0	0.0
Together	11.10±0.81	10.80±0.67	10.32±0.83	9.72±0.77	11.88±0.81

Note: Cell entries are mean number of occurrences ±one S.E.M. in morphine treated animals.

Discussion

These data show that the administration of the hydrophilic opiate antagonist methylnaloxonium into the PAG or LC, but not the amygdala elicited clear signs of opiate withdrawal. Withdrawal induced after administration of methylnaloxonium into the LC and PAG of morphine dependent 7 day-old rats was similar to that reported after systemic administration of naltrexone in the 7 day-old rat (Jones, & Barr, 1995). There were interesting differences between the behaviors elicited by PAG and LC stimulation. Antagonist injection to the PAG elicited intense motor signs, such as wall climbing, rolling, walking, and less time spent quiet. Injection of the LC elicited similar gross motor signs such as wall climbing, head movements, paw movements and less time quiet, but only weakly elicited rolling and walking. The most notable difference between the two sites was the continuous chewing movement and the loud and clearly audible vocalization that was "chirp-like" in nature observed following administration of methylnaloxonium into the LC of morphine dependent animals. These behaviors were not observed following methylnaloxonium injection into the PAG of morphine dependent animals. It should also be noted that these behaviors were not observed following peripheral injections (Jones & Barr, 1995; Windh, et al.,1995).

In the adult rat, as in the infant the most sensitive site for methylnaloxonium-precipitated withdrawal is the LC (Maldonado, et al., 1992). Signs such as jumping, rearing, teeth chattering, chewing, and locomotor activity were particularly frequent after methylnaloxonium injections into the adult LC (Maldonado, et al., 1992). The LC represents the largest cluster of noradrenergic neurons in the brain and contains a high density of opioid receptors, particularly of the μ type. A noradrenergic hyperactivity in the LC has been hypothesized to mediate the expression of some components of the physical morphine withdrawal (Maldonado & Koob, 1993). For example, there is an increase in the noradrenergic neuronal firing rate in the LC during naloxone-precipitated morphine withdrawal that shows a similar time course to that of the behavioral signs of withdrawal (Aghajanian, 1978); destruction of the LC decreases physical signs of opiate withdrawal (Maldonado, & Koob, 1993); and local infusions of the α_2 agonist clonidine, which suppresses withdrawal-induced activation of LC neurons (Aghajanian, 1978), attenuates both the behavioral (Taylor, et al., 1988), and biochemical changes associated with opiate withdrawal (Laverty, & Roth, 1980). Additionally, stimulation of the LC produces several behavioral signs of morphine withdrawal (Rasmussen, et al., 1990). Studies of local cerebral glucose utilization have shown significantly enhanced metabolic activity in the LC during

naloxone precipitated withdrawal in the adult rat (Kimes & London, 1989) and there is also an increase in the staining of the nuclear proto-oncogene c-fos in the LC during opiate withdrawal in awake and anesthetized morphine-dependent rats (Chieng, et.al., 1995; Hayward, et al., 1990).

With the exception of the present study, there are no available data addressing the possible role of the LC in the development of morphine dependence in the neonatal animal. What we do know is that μ opioid receptors can be detected gestational in the CNS (Kornblum et al., 1987) and relatively high levels of mu receptors are present in the LC. Additionally, LC neurons in neonates have little or no spontaneous activity, but are sensitive to both noxious and non-noxious sensory stimuli (Nakamura, & Sakaguchi, 1990). Nakamura's group has also shown that antidromic activation of the LC from stimulation of the cortex can be detected as early as embryonic day 18 (Nakamura and Sakaguchi, 1990). Furthermore, the LC is considered to be implicated in vigilance, arousal and in the hyperemotional behavior associated with anxiety, stress and fear (Abercrombie, & Jacobs, 1987; Aston-Jones, et al., 1985) and, accordingly, the opiate withdrawal syndrome in human infants is characterized by symptoms such as high-pitched crying, increased muscle tone, irritability, and altered sleep and waking patterns. All of these studies taken together suggest, although indirectly, that the LC plays a

major role in the development of opiate withdrawal in the neonatal rat.

The administration of methylnaloxonium into the PAG also elicits a severe withdrawal syndrome in the neonatal rat. This finding parallels with studies in the adult rat where methylnaloxonium injection into the PAG precipitates a strong withdrawal syndrome characterized by behaviors such as teeth chattering, chewing, jumping, rearing and hyperactivity. Rearing and hyperactivity were particularly elevated (Maldonado et al., 1992). The general importance of the periaqueductal gray (PAG) in the development of opiate dependence and the expression of physical withdrawal in the adult rat has been further supported in the past, first by the presence of a strong withdrawal syndrome following administration of opiate antagonist (naloxone and levallorphan) into the PAG (Laschka, et al., 1976) as well as by the severe physical dependence observed after chronic administration of enkephaline analogs (Wei, et al., 1974) and morphine (Bozarth and Wise, 1984) into this region. More recently, metabolic activity has been shown to be significantly enhanced in the PAG of adult animals during naloxone-precipitated withdrawal (Kimes & London, 1989) and increased c-fos-like immunoreactivity in neurons of the ventrolateral PAG are significantly increased during naloxone-precipitated withdrawal (Chieng et al., 1995). The PAG has also been shown to be involved in other opiate mediated functions, notably analgesia (Sharpe et al., 1974;

Jacquet & Lajtha, 1976). Although the administration of methylnaloxonium into the PAG elicits a severe withdrawal syndrome (Maldonado et al., 1992), it is not very sensitive to the methylnaloxonium-induced aversive effects of opiate abstinence (Koob et al., 1989; Stinus, et al., 1990). For example, local injections of methylnaloxonium into the PAG does not elicit a disruption in food operant responding in the adult rat (Koob, et. al., 1989) and fails to elicit place aversions (Stinus, et. al., 1990). Although autoradiographic studies have indicated that opiate receptors are present at significant densities in the PAG at birth, with the exception of the present study, there are no available data addressing the possible involvement of the PAG in the neonatal opiate withdrawal syndrome. Based on the results of the present study it seems likely that as in the adult rat, the PAG is critically involved in the development of physical dependence and in the expression of physical withdrawal to morphine in the neonatal rat.

In the present study, there were no behavioral changes following stimulation of the amygdala. Although physical signs of withdrawal from the amygdala in the adult are substantially milder than the PAG or LC, they do occur in the adult rat. Whereas earlier studies have reported a significant withdrawal syndrome, especially an increase in the jumping behavior, following injection of naloxone into the amygdala of the adult (Calvino, et al., 1979; Tremblay, &

Charton, 1981), more recent studies injecting methylnaloxonium into the amygdala induced only a very mild withdrawal in which jumping, wet dog shakes, and other major signs of abstinence were absent even when the antagonist was administered at the highest dose (1000 ng) (Maldonado, et al., 1992). Maldonado's group attributes this discrepancy to a greater diffusion of naloxone in the brain. However, a significant incidence of teeth chattering and chewing were observed following antagonist injection into the amygdala of morphine dependent animals (Maldonado, et al., 1992). Chewing is also seen following antagonist injection to the LC in both the infant and adult rat; yet there was no evidence of chewing in the pup following antagonist injection to the amygdala. This suggest a differential maturation of the expression of similar behaviors. On the other hand, the amygdala has been shown to be an important anatomical site for mediating the aversive affect of opiate withdrawal. For example, low doses of methylnaloxonium into the amygdala disrupted operant responding for food and produced place aversions in morphine dependent adult rats but failed to elicit overt signs of withdrawal (Koob, et al., 1989; Stinus, et al., 1990; Wei, et al., 1973). Furthermore, small lesions of the dorsomedial nuclei of the amygdala had no effect on the naltrexone-precipitated morphine withdrawal symptoms, but they did decrease the magnitude of the conditioned place aversion produced by that

withdrawal in the adult rat (Kelsey, & Arnold, 1994). The poor correlation between the expression of the physical signs of opiate withdrawal and the aversive stimulus properties of the opiate withdrawal syndrome suggests a different neuroanatomical substrate for these two processes in the adult rat. Whether or not the amygdala is involved in the aversive aspects of withdrawal in the infant animal remains to be investigated.

Recently, our lab has described the maturation of the physical signs of opiate withdrawal from the fetus (day 20 of gestation) through 42 days of postnatal life (Jones & Barr, 1995; Jones & Barr, submitted). At all stages of development, precipitated withdrawal was evidenced by a distinct and consistent behavioral syndrome, but the nature of that syndrome changed with age. Adult-like responses, which had been studied previously, did not appear until puberty. Similar findings in the week old pup was published at virtually the same time by Kuhn's group (Windh, et al., 1995). The recent description of the consequences of opiate withdrawal during development has laid the foundation for the elucidation of the anatomical sites involved in the opiate withdrawal syndrome in the infant. There is no reason to assume that the same sites in the adult mediate the withdrawal syndrome in the infant. Indeed, one hypothesis for the different adult and infant constellations of withdrawal signs is that they are mediated by

separate neural circuits and that the maturation of the adult withdrawal syndrome depends on the decreasing role of the neural circuits for infant abstinence and the increased involvement of neural systems involved in the adult syndrome. The alternative hypothesis is the same neural sites mediate withdrawal throughout the life span, but the effector systems change. For example, in many species, including the rat, and to a somewhat lesser extent, humans, sympathetic innervation of autonomic end-organs is absent or non-functional at birth (1988a,b). In the adult, increased stimulation of postsynaptic receptor sites produces compensatory desensitization that reduces tissue responsiveness. During development, however, responses in most systems increase with age and with the maturation of neuronal inputs (Giannuzzi, et al., 1995). This group has shown that regulation of receptor signaling is completely different early in neonatal life. Instead of producing desensitization of responses, agonist exposure promotes receptor signaling by enhancing expression and/or catalytic efficiency of adenylyl cyclase. In older animals, the predominant effect is heterologous desensitization mediated at the level of G-proteins. These developmental differences are likely to be important in the maintenance of tissue responsiveness during the period in which innervation develops, as well as in the ability of neurotrophic input to "program" the responsiveness of target tissues (Giannuzzi, et al., 1995).

Furthermore, it has been shown that catecholamines released by the adrenal medulla during birth play a key role in the adaptation of the newborn to extrauterine life. Respiratory, metabolic, and cardiovascular adaptations to hypoxia and other stresses associated with delivery are dependent upon a profound surge of adrenomedullary activity which occurs despite the immaturity of connections between the CNS and the adrenal (Slotkin & Seidler, 1988a). The "non-neurogenic" response seen in the fetus and neonate is thus essential to survival, and any interference with catecholamine actions at adrenergic targets results in loss of the ability to survive hypoxia or other stressors. The immature secretory mechanism disappears as a result of development of neural connections, and factors which accelerate ontogeny of neural competence thus increase vulnerability. The ontogenetic "switchover" of receptor-mediated mechanisms appears to be a functioning of the development of neuronal competence (Slotkin & Seidler, 1988a, 1988b).

In conclusion, it seems likely that the same neural circuitry mediates physical withdrawal at different ages, regardless of the specifics of the behaviors elicited. Our results suggests that the neural circuitry that initiates withdrawal matures early, but "down-stream" effector systems (e.g. autonomic nervous system; motor systems) are later developing. The differences suggest differential

maturation of certain neural mediators of withdrawal.

Chapter 5

General Discussion

Although the neonatal abstinence syndrome is a contemporary terminology, newborn drug withdrawal has been a known consequence of opiate dependency in pregnant women for more than a century. A review of the early literature provides a historical perspective to the field of perinatal opioid dependence. As early as 1895 a group of investigators found that morphine could pass through the human placenta (Bureau, 1985). Even the absorption of morphine into breast milk was clearly understood and well documented (Laase, 1919; Langstein, 1930; Petty, 1912, 1913; Van Kleek, 1920). Moreover, the long-term ramifications of maternal opiate abuse for her infant was also published by these early investigators (Wilson & Eshner, 1896).

The 1950's and 1960's was a period of time in which there was an increase in the number of women of childbearing age using heroin. The bulk of research during this time was clinical in nature focusing on the identification of the effects of opiates on the developing fetus as manifested in the neonate. The emphasis was centered on the recognition and pharmacological management of the neonatal abstinence syndrome which eventually led to the development of a neonatal abstinence scoring system to assess the onset, progression, and delineation of symptoms (Finnegan, 1976).

The 1970's was distinguished by the implementation of methadone

maintenance for opiate dependence in pregnancy, the rationale being that a longer lasting opiate dose might minimize opiate withdrawal in the infant (Kaltenbach & Finnegan, 1992). The emphasis was then shifted to the need to identify potential risks to the offspring of pregnant opiate-dependent women. Numerous, researchers reported on methadone's interaction in the fetus as well as the neonate (Wallach et al., 1969; Zelson, & Casalino, 1973) which today continues to be an area of intense research.

Although the literature describing the opiate withdrawal syndrome in the human infant is well described, it is plagued with interpretive problems that are nearly intractable. Within the past several years perinatal addiction studies have been characterized by the acknowledgment that not only biological but social and environmental factors related to maternal drug abuse may be significant confounding factors affecting infant outcome (Hutchings, 1990; Kaltenback & Finnegan; 1992; Kaltenback, 1994). These factors include: low socioeconomic status, often associated with psychiatric and medical problems, poor diets and inadequate prenatal care; and multidrug use associated with the concurrent use of marijuana, cocaine, tranquilizers or alcohol (Hutchings, 1990). In order to examine the effects of opiate withdrawal in the absence of these confounding problems, it is necessary to turn to an animal model. Historically, by the time

developmental research using animal models is initiated on a given drug, a considerable amount of data exists describing its interactions in the adult. Opiate drugs are certainly no exception to this rule. In the late 1970's and 1980's an abundance of research was published describing the behavioral manifestations of the opiate withdrawal syndrome in the adult rat. These data taken together have provided an animal model to investigate further the neurobiological basis of opiate dependence. Thus, this animal model has become an effective tool in describing not only the behavioral aspects of the opiate withdrawal syndrome but more recently, the anatomical and cellular mechanisms that mediate this syndrome in the adult animal. Additionally, this model has served as a guide in that it has provided some fundamental knowledge to aid in the designing of experiments that address the issue of opiate dependence in the developing animal (Jones & Barr, 1995, Windh, et al., 1995).

Prior to this thesis, the existence of a fetal and neonatal withdrawal syndrome had only been hypothesized in animal models based on teratologic studies (Kuwahara & Sparber, 1981; Kirby, 1981b). Although previous studies have supported the argument that key components of a functional opioid system, including endogenous opioids and opioid receptors, are present during the prenatal period and by birth (Spain, et al., 1985; Kirby, 1981a), few researchers focused on

withdrawal effects in neonatal animal studies. There are several possible explanations for this: First, the neurochemical pathways underlying withdrawal in the adult rat have only very recently been described, providing a clearer understanding of the anatomical and cellular components that mediate withdrawal in the adult. Secondly, it is possible that the absence of adult-like physical withdrawal signs in the infant rat has led investigators to assume that a withdrawal syndrome does not occur in the infant.

The experiments of this thesis were therefore designed to construct a developmental animal model to allow further investigation of the opiate withdrawal syndrome in the developing animal. The hypothesis of this thesis was that both the fetus and infant are capable of experiencing opiate withdrawal when the age specific behavioral repertoire to the age of the animal is examined. The goal of this dissertation was therefore to describe in detail the unconditioned behavioral changes that follow precipitated withdrawal from chronic exposure to morphine during development, including the fetus, the neonate, and continuing through adolescence and to define specific anatomical mechanisms that may mediate the withdrawal syndrome in the infant rat.

This was a 2-stage process. In the first stage, the dam or the rat pup was treated chronically with morphine and injections of naltrexone were given to the

fetus or pup to precipitate withdrawal. The first experiment of this thesis provides an extensive description of the maturation of precipitated withdrawal from 7 to 42 days of age. At all stages of development, precipitated withdrawal was evidenced by a distinct and consistent behavioral syndrome, but the nature of that syndrome changed with age. Indeed adult-like responses which had been studied previously did not appear until puberty. Three major developmental patterns of withdrawal emerged. There were abstinence behaviors that were unique to the 7 or 14 day old pup. These included head swaying, hindpaw movements, rolling, and stretching. Other behaviors appeared only in the 21 or 42 day old pup. These behaviors were burrowing, diarrhea, jumping, teeth chattering, and wet dog shakes and constitute the adult withdrawal syndrome. Finally, there were behaviors labeled developmentally continuous behaviors that were characteristic of animals in withdrawal at all ages. These included walking forward and failure to be quiet. The results of this study indicate that morphine abstinent rats demonstrate withdrawal behaviors that are within the developmental repertoire of the animal thereby supporting the hypothesis of this thesis.

After completing the postnatal description of withdrawal in the rat, the focus was shifted to the development of the physical signs of withdrawal in the fetus (day 20 of gestation). The results of this study show that naltrexone-injected

GD 20 fetuses that had been passively exposed to morphine (through the placental transport of the drug) show specific alterations in behavior, consisting of increased body curls, limb and mouth movements, face wiping, and less quiet time as compared to control animals. Other behaviors such as body twitching, head movements, and stretching did not change. An interesting result of this experiment was that the behavior face wiping only occurred in morphine exposed animals treated with naltrexone. Face wiping is a behavior previously described in the fetal animal as a pattern of motor behavior rarely seen in the absence of aversive stimulation (Smotherman & Robinson, 1992). These findings taken together suggest that face wiping may be a motor response evoked by aversive stimuli unique to the fetus. Furthermore, this study describes a set of behaviors that characterize opiate withdrawal *in utero*.

The quantification of specific behavioral changes unique to precipitated withdrawal in the fetal and infant animal was a necessary step to understand further the mechanisms of morphine dependence in the developing animal. Together the first two experiments of this thesis indicate that the fetus and infant do experience withdrawal from opiates suggesting the possibility that abstinence may be in part responsible for some of the adverse effects of opiates observed in animals as well as humans. Furthermore, these experiments provided a developmental model to

study the mechanisms of drug dependence as they change through development, thereby setting the stage for the elucidation of the anatomical and physiological substrates that mediate this syndrome at different levels of development.

Therefore, using the first set of experiments as a model, the goal of the second stage of this thesis was to examine the role of specific neural sites in the precipitated withdrawal syndrome in the 7 day old rat, concentrating on three brain regions, the amygdala, the periaqueductal gray (PAG), and the locus ceoruleus (LC). These sites were chosen because they are sites known to be involved in the morphine withdrawal syndrome in the adult animal. In the adult, opiates produce their biological actions at specific brain regions, and likewise the various aspects of the opiate withdrawal syndrome are mediated through identified brain loci that are spread from the prefrontal cortex to the spinal cord. Even though we chose common brain sites involved in the adult withdrawal syndrome, one should not assume that these same sites in the adult mediate withdrawal in the infant animal. Indeed, one hypothesis for the different adult and infant constellations of withdrawal signs is that they are mediated by separate neural circuits and that the maturation of the adult withdrawal syndrome depends on the decreasing role of the neural circuits for infant abstinence and the increased involvement of neural systems involved in the adult syndrome. The alternative hypothesis is the same

neural sites mediate withdrawal throughout the life span, but the effector systems change (e.g. the autonomic nervous system, motor output). The results of the final study of this thesis suggest that the latter hypothesis is correct. The results showed that the administration of methylnaloxonium into the periaqueductal gray of the midbrain or the locus ceoruleus region produced a withdrawal syndrome similar to that elicited by peripheral injection of naltrexone in the 7 day-old rat, but different than that of the adult. Thus, it seems likely that the same neural circuitry mediates physical withdrawal at different ages, regardless of the specifics of the behaviors elicited.

Of the neurobehavioral effects that can be produced by opiate drugs, some of the most serious by far are the complex set of symptoms that comprise the neonatal abstinence syndrome. The results of these experiments therefore provides some valuable data that were not previously available, particularly the detailed description of the behavioral components that characterize the opiate withdrawal syndrome at different stages of development. These experiments are also unique in that they provide a developmental model to study the neural mechanisms of drug dependence as they change throughout development. A complete understanding of these mechanisms will increase the likelihood of the development of pharmacological agents that might possibly prevent or reverse the actions of

opiates on specific target neurons as well as treat the physical symptoms associated with the opiate withdrawal syndrome in the human infant.

References

Abdelhamid, E. E., & Takemori, A.E. (1991). Characteristics of μ opioid binding sites in striated slices of morphine tolerant and dependent mice. *European Journal of Pharmacology*, 198, 157-163.

Abercrombie, E.D., & Jacobs, B.L. (1987). Single-unit response of noradrenergic neurons in the locus coeruleus axons of freely moving cats I. Acutely presented stressful and nonstressful stimuli. *Journal of Neuroscience*, 7, 2837-2843.

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

Adler, M.W., Geller, E.B., Beeton, P.B., & Gildenberg, P.L. (1978). Inability of acute or chronic thalamic, limbic, or cortical lesions to alter narcotic dependence and abstinence in rats. *Developmental Neuroscience*, 4, 51-52.

Advokat, C. (1981). Environmental modulation of analgesic tolerance induced by morphine pellets. *Pharmacology, Biochemistry, & Behavior, 14*, 139-142.

Aghajanian, G.K., (1978). Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal response by clonidine. *Nature*, 267, 186-187.

Akaoka, H., & Aston-Jones, G. (1991). Opiate withdrawal-induced hyperactivity of locus coeruleus neurons is substantially mediated by augmented excitatory amino acid input. *Journal of Neuroscience*, 11, 3830-3839.

Allin, J.T. & Banks, E.M. (1972). Effects of temperature on ultrasound production by infant albino rats. *Developmental Psychobiology, 4*, 149-156.

Amsel, A., Radek, C.C., Graham, M. & Letz, R. (1977). Ultrasound emission in infant rat as an indicant of arousal during appetitive learning and extinction. *Science, 197*, 786-788.

Andrade, R., VanderMaelen, C.P., & Agajanian, G.K. (1983). Morphine tolerance and dependence in the locus coeruleus: single cell studies in brain slices. *European Journal of Pharmacology*, 91, 161-169.

Aston-Jones, G., Foote, S.L., & Segal, M. (1985). Impulse conduction properties of noradrenergic locus coeruleus axons projecting to monkey cerebrocortex. *Neuroscience*, 15, 765-777.

Barr, G.A., & Goodwin, G.A. (1997). Precipitated morphine withdrawal induces a conditioned aversion in the preweaning rat. *Pharmacology, Biochemistry, & Behavior*, In Press.

Barr, G.A. & Wang, S. (1992). Tolerance and withdrawal to chronic morphine treatment in the week-old rat pup. *European Journal of Pharmacology, 215*, 35-42.

Bell, R.W. (1971). Ultrasounds in small rodents: Arousal-produced and arousal-producing. *Developmental Psychobilogy, 7*, 39-44.

Besunder, J.B., & Blumer, J.L. (1990). Neonatal drug withdrawal syndromes. From Maternal-Fetal Toxicology. (ed) Gideon Koren Marcel Pekker, Inc. 161-177.

Bhagrava, H.N., & Gulati, A. (1990). Down-regulation of brain and spinal cord μ-opiate receptors in morphine tolerant-dependent rats. *Journal of Pharmacology*, 190, 305-311.

Blasig, J., Herz, A., Reinhold, K., & Zieglgansberger, W.S. (1973). Development of physical dependece on morphine in respect to in rats. *Psychopharmacology, 33*, 19-38.

Blatman, S., & Lipsitz, P.J. (1972). Children of women maintained on methadone: accidental methadone poisoning of children. In: Proceedings of the Fourth National Conference on Methadone Treatment, NAPAN, New York, 175-176.

Blinick, G. (1968). Menstrual function and pregnancy in narcotic addicts treated with methadone. *Nature*, 219, 180.

Boddy, K., Dawes, G.S., Fisher, R., Pinter, S., & Robinson, J.S. (1974). Foetal respiratory movements, electrocortical and cardiovascular responses to hypoxia and hypercapnia in sheep. *Journal of Physiology*, 243, 599-618.

Bozarth, M.A., & Wise, R.A. (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science*, 224, 516-517.

Brady, L.S., Herkenham, M., Long, J.B., & Rothman, R.B. (1989). Chronic morphine increases μ-opiate receptor binding in rat brain: A quantitative autoradiographic study. *Brain Research*, 477, 386.

Bureau, A. (1895). Accouchement d'une morphinomane: prevue chimique du passage de la morphine a travers le placenta: reflexions. *Bull Mem. Soc. Obstet. Gynecol.*,356-362.

Calvino, B., Lagowska, J, & Ben-Ari, Y. (1979). Morphine withdrawal syndrome: differential participation of structures located withing the amygdaloid complex and striatum of the rat. *Brain Research*, 177, 19-34.

Chang, S.L., Squinto, S.P., & Harlan, R.E. (1988). Morphine activation of c-fos expression in rat brain. *Biochemical and biophysical research communications*, 157, 698-704.

Chasnoff, I.J. (1988). Drug use in pregnancy: parameters of risk. *Pediatric Clinics of North America*, 35, 1403-1412.

Chen, Y., Mestek, A., Liu, J., Hurley, J.A., & Yu, L. (1993). Molecular cloning and functional expression of μ-opioid receptor from rat brain. *Molecular Pharmacology*, 44, 8-12.

Cicero, T., & Meyer, E.R. (1973). Morphine pellet implantation in rats: Quantitative assessment of tolerance and dependence. *Journal Pharmacology & Experimental Therapeutics, 184*, 404-408

Chieng, B. & Christie, M.J. (1995). Lesions to terminals of noradrenergic neurones do not inhibit opiate withdrawal behavior in rats. *Neuroscience Letters*, 186, 37-40.

Chieng, B., Keay, K.A., & Christie, M.J. (1995). Increased fos-like immunoreactivity in the periaqueductal gray of anaesthetised rats during opiate withdrawal. *Neuroscience Letters*, 183, 79-82.

Childers, S.R. (1993). Opioid receptor-coupled second messenger systems. *Handbook of Experimental Pharmacology*, 104, 189-216.

Christie, M.J., Williams, J.T., & North, R.A., (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Molecular Pharmacology*, 32, 633-638.

Clendeninn, N.J., Petraitis, M., & Simon, E.J. (1976). Ontological development of opiate receptors in rodent brain. *Brain Research*, 118, 157-160.

Cohen, M.S., Rudolph, A.M., & Melmon, K.L. (1980). Antagonism of morphine by naloxone in pregnant ewes and fetal lambs. *Developmental Pharmacology Therapeutics, 1*, 58-69.

Cox, B.M. (1993). Opioid receptor-Gprotein interactions: acute and chronic effects of opioids. *Handbook of Experimental Pharmacology*, 104, 145-188.

Desmond, M.M., & Wilson, G.S. (1975). Neonatal abstinence syndrome: recognition and diagnosis. *Addictive Disorders*, 2, 113-121.

Dinges, D.F., Davis, M.M., & Glass, P. (1980). Fetal exposure to narcotics: neonatal sleep as a measure of nervous system disturbance. *Science*, 209, 619-621.

De Vries, T.S., Tjon Tien Ril, G.H.K., Van Der Laan, J.W., Mulder, A.H., Schoffelmeer, A.N.M. (1993). Chronic exposure to morphine and naltrexone induces changes in catecholaminergic neurotransmission in rat brain without altering μ-opioid receptor sensitivity. *Life Sciences*, 52, 1685-1693.

Dube, S.K. (1981). Urinary catecholamines, plasma activity and blood pressure in newborns: Effects of narcotic withdrawal. *Developmental Pharamacology and Therapeutics*, 3, 83-87.

Ennis, M., & Aston-Jones, G. (1988). Activation of locus coeruleus from nucleus paragiganto-cellularis: a new excitatory amino acid pathway in brain. *Journal Neuroscience*, 8, 3644-3657.

Evans, C.J., Keith, D.E., Jr., Morrison, H., Magendzo, K., & Edwards, R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science*, 258, 1952-1955.

Fanselow, M.S., & Cramer, C.P. (1988). The ontogeny of opiate tolerance and withdrawal in infant rats. *Pharmacology, Biochemistry &Behavior, 31*, 431-438.

Finnegan, L.P. (1976). Clinical effects of pharmacologic agents on pregnancy, the fetus and neonate. *Annals of the New York Academy of Science, 281*, 74-89.

Franck, L. & Vilardi, J., (1995). Assessment and management of opioid withdrawal in ill neonates. *Neonatal Network*, 14, 39-45.

Geary, W.A., II, & Wooten, G.F. (1983). A behavioral and 2-deoxyglucose autoradiographic study of the effects of cumulative morphine dose on naloxone precipitated withdrawal in the rat. *Brain Research*, 275, 117-126.

Geary, W.A., II, & Wooten, G.F. (1984). Similar functional anatomy of spontaneous and precipitated morphine withdrawal. *Brain Research*, 334, 183-186.

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

Geary, W.A. II, & Wooten, G.F. (1985b). Similar functional anatomy of spontaneous and precipitated morphine withdrawal. *Brain Research*, 334, 183-186.

Geary, W.A., II, & Wooten, G.F. (1986). Time action profiles of regional glucose utilization during naloxone precipitated morphine withdrawal. *Brain Research*, 399, 181-184.

Giannuzzi, C.E., Seidler, F.J., & Slotkin, T.A. (1995). Beta-adrenoreceptor control of acardiac adrenylyl cyclase during development: agonist pretreatment in the neonate uniquely causes heterologous sensitization, not desensitization. *Brain Research*, 694, 271-8.

Glick, S.D., & Charap, A.D. (1973). Morphine dependence in rats with medial forebrain bundle lesions. *Psychopharmacology, 30*, 343-348.

Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapiller, M., Hood, L. (1979). Dynorphin (1-13), an extraordinarily potent opioid peptide. *Poceedings of the National Academy of Science, USA*, 76, 6666-70.

Guitart, X., & Nestler, E.J. (1990). Identification of MARP-14-20, morphine-and cyclic AMP-regulated phosoproteins of 14-20 kD, as myelin basic proteins. Evidence for their acute and chronic regulation by morphine in rat brain. *Brain Research*, 516, 57-65.

Guyenet, P.G., & Young, B.S. (1987). Pojections of nucleus paragigantocellularis lateralis to locus coeruleus and other structures in rat. *Brain Research*, 406, 171-184.

Haertzen, C.A., & Hoobs, N.T., Jr. (1969). Changes in personality (MMPI) and subjective experience (ARCI) throughout a cycle of addiction to morphine. *Journal of Nervous Mental Disorders*, 148, 606-614.

Hans, S.L. Marcus, J., Jeremy, R.R., & Auerbach, J.G. (1984). Neurobehavioral development of children exposed *in utero* to opioid drugs. From Neurobehavioral Teratology, (ed) Joseph Yanai, Elsevier, 249-273.

Hayward, M.D., Duman, R.S., & Nestler, E.J. (1990). Induction of the *c-fos* proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Research*, 525, 256-266.

Henningfield, J.E., Johnson, R.E., & Jasinski, D.R. (1987). Clinical procedures for the assessment of abuse potential. In methods of Assessing the Reinforcing Properties of Abused Drugs, ed. By Bozarth, M.A. Springer-Verlag, New York, 573-590.

Hodding, G.C., Jann, M., & Ackerman, I.P. (1980). Drug withdrawal syndromes: A literature review. *Western Journal of medicine*, 133, 383-391.

Hofer, M.A., & Shair, H. N. (1987). Isolation distress in 2 week old rats: Influence of home cage, social companions and prior experience with littermates. *Developmental Psychobiology, 20*, 465-476.

Hutchings, D.E. (1990). Issues of risk assessment: lessons from the use and abuse of drugs during pregnancy. *Neurotoxicology & Teratology 12*, 183-189.

Jackler, F., Steiner, S.S., Bodnar, R.J., Ackerman, R.F., Nelson, W.T., & Ellman, S.J. (1979). Morphine and intracranial self-stimulation in the hypothalamus and dorsal brainstem; differential effects of doses, time and site. *International Journal of Neuroscience, 9*, 21-35.

Jacquet, Y.F., & Lajtha, A. (1976). The periaqueductal gray: site of morphine analgesia and tolerance as shown by 2-way-cross-tolerance between systemic and introcerebral injections. *Brain Research*, 103, 501-513.

Jaffe, J.H., & Martin, W.R. (1980). Opioid analgesics and antagonists. In: Gilman A.G., Goodman, L.S., Gilman, A. (Eds) Goodman and Gilman's The Pharmacological Basis of Therapeutics, New York, Macmillan, 535-584.

Jaffe, J. H. (1990). Drug addiction and drug abuse. In Goodman & Gillman's The Pharmacological Basis of Therapeutics, 8th ed, Gilman, A. Et al., eds. New York: Pergamon press, 523-573.

Jones, K.L., & Barr, G.A. (1995). Ontogeny of morphine withdrawal in the rat. *Behavioral Neuroscience, 109, 6*, 1189-1198.

Kaltenbach, K.A. & Finnegan, L. (1992). Prenatal opiate exposure: Physical, neurobehavioral, and developmental effects. Development of the Central Nervous System: Effects of Alcohol and Opiates, 37-46.

Kaltenbach, K.A. (1994). Effects of in-utero opiate exposure: new paradigms for old questions. *Drug and Alcohol Dependence*, 36, 83-87.

Kandel, E.R., Schwartz, J.H., & Jessell, T.M. (1991). Principles of Neural Science. 3rd Edition. Norwalk, Connecticut: Appleton & Lange, 123-258.

Kelsey, J.E., & Arnold, S.R. (1994). Lesions of the dorsomedial amygdala, but not the nucleus accumbens, reduce the aversiveness of morphine withdrawal in rats. *Behavioral Neuroscience*, 108, 1119-1127.

Kerr, F.L., & Pozuilo, J. (1971). Suppression of physical dependence and induction of hypersensitivity to morphine by stereotaxic lesions in addicted rats. *Proceedings of the Mayo Clinic*, 46, 653-655.

Kieffer, B.L., Befort, K., Gaveriaux-Ruff, C., Hirth, C.G. (1992). The δ-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. *Proceedings of the National Academy of Science USA*, 89, 12048-12052.

Kimes, A.S. & London, E.D. (1989). Glucose utilization in the rat brain during chronic morphine treatment and naloxone-precipitated morphine withdrawal. *Journal of Pharmacology and Experimental Therapeutics*, 48, 535-545.

Kirby, M.L., (1979). Effects of morphine on spontaneous activity of 18-day old rat fetuses. *Developmental Neuroscience, 2*, 23-244.

Kirby, M.L. (1981a). Effects of morphine & naloxone on spontaneous activity of fetal rats. *Experimental Neurology, 73*, 430-439.

Kirby, M.L. (1981b). Development of opiate receptor binding in rat spinal cord. *Brain Research*, 205, 400-404.

Kirby, M.L., & Aronstam, R.S. (1983). Levorphanol-sensitive [^{3}H] naloxone binding in developing brainstem following prenatal morphine exposure. *Neuroscience Letters, 35*, 191-195.

Koob, G.F., Wall, T.L., & Bloom, F.E. (1989). Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal. *Psychopharmacology*, 98, 530-534.

Koos, B.J., Sameshima, H., & Power, G.G. (1987). Fetal breathing, sleep state, and cardiovascular responses to graded hypoxia in sheep. *Journal of Applied Physiology*, 63, 1033-1039.

Kornblum, H.I., Hurlbut, D.E., & Leslie, F.M. (1987). Postnatal development of multiple opioid receptors in rat brain. *Developmental Brain Research*, 37, 21-41.

Kuwahara, M.D., & Sparber, S.B. (1981). Prenatal withdrawal from opiates interferes with hatching of otherwise viable chick fetuses. *Science, 212*, 945-947.

Laase, C.F.J. (1919). Narcotic drug addiction in the newborn, report of a case. *American Medicine*, 25, 283-286.

Langstein, L. (1930). Uber das schicksal von morphiumsuchtigen frauen geborener sauglinge. *Med. Klin.*, 26, 500-501.

Laschka, E., Teschemacher, Hj., Mehraein, P., & Herz, A. (1976). 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, 141-147.

Laschka, E., & Herz, A. (1977). Sites of action of morphine involved in the development of physical dependence in rats III. Autoradiographic studies. *Psychopharmacology*, 53, 33-37.

Laverty, R., & Roth, R.H. (1980). Clonidine reverses the increased norepinephrine turnover during morphine withdrawal in rats. *Brain Research*, 182, 482-485.

Lichtblau, L., & Sparber, S.B. (1981). Opiate withdrawal *in utero* increases neonatal morbidity in the rat. *Science, 212*, 943-945.

Linseman, M. (1977). Naloxone-precipitated withdrawal as a function of the morphine-naloxone interval. *Psychopharmacology, 54*, 159-164.

Loh, H.H., & Smith, A.P. (1990). Molecular characterization of opioid receptors. *Annual Review Pharmacology & Toxicology*, 30, 123-147.

Mackler, S.A., & Eberwine, J. H. (1994). Cellular adaptation to opiates alters ion-channel mRNA levels. *Proc. Natl. Acad. Sci. USA*, 91, 385-389.

Maldonado, R., Stinus, L., Gold, L.H., & Koob, G.F. (1992). Role of different brain structures in the expression of physical morphine withdrawal syndrome. *Journal of Pharmacology & Experimental Therapeutics*, 261, 669-677.

Maldonado, R. & Koob, G.F. (1993). Destruction of the locus coeruleus decreases physical signs of opiate withdrawal. *Brain Research*, 605, 128-138.

Mannering, F.J., & Jackson, M.C. (1977). Enduring effects of morphine pellets revealed y conditioned taste aversion. *Psychopharmacology*, 51, 279-283.

Meng, F., Xie, G.X., Thompson, R.C., Mansour, A., Goldstein, A., Watson, S.J., & Akil, Huda. (1993). Cloning and pharamacological characterization of a rat κ opioid receptor. *Proc. Natl. Acad. Sci. USA*, 90, 9954-9958.

Mucha, R.F., Van der Kooy, D., O'Shaughnessy, M., & Bucenieks, P. (1982). Drug reinforcement studied by the use of place conditioning in rat. *Brain Research, 243*, 91-105.

Mucha, R.F. (1987). Is the motivation effects of opiate withdrawal reflected by common somatic indices of precipitated withdrawal? A place conditioning study in the rat. *Brain Research, 418*, 214-247.

Mucha, R.F. (1991). What is learned during opiate withdrawal conditioning? Evidence for a cue avoidance model. *Psychopharmacology, 104*, 391-396.

Nakamura, S., & Sakaguchi, T. (1990). Development and plasticity of the locus coeruleus: a review of recent physiological and pharmacological experimentation. *Prog. Neurobiology*, 34, 505-526.

Naeye, R.L. (1965). Malnutrition, a probable cause of fetal growth retardation. *Arch. Pathol., 79*, 284.

National Institutes of Health (1985). Guide for care & use of laboratory animals (U.S. Public Health Service Publication No. 86-23). Washington, D.C. U.S. Government Printing Office.

Nestler, E.J. (1992). Molecular mechanisms of drug addiction. *Journal of Neuroscience, 12,* 2439-2450.

Neumann, L.L., & Cohen, S.N. (1978). The neonatal narcotic withdrawal syndrome: a therapeutic challenge. *Clinical Perinatology,* 2, 99-109.

Nishino, K., Su, Y.F., Won, C.S., Watkins, W.D., & Chang, K.J. (1990). Dissassociation of mu opioid tolerance from receptor downregulation in rat brain spinal cord. *Journal of Pharmacology & Experimental Therapeutics,* 253, 67-72.

Noirot, E. (1972). Ultrasounds and maternal behavior in small rodents. *Developmental Psychobiology, 5,* 371-388.

O'Brien, C.P., Childers, A.R., Arnt, I.O., McLellan, T., Woody, G.B., & Maany, I. (1988). Pharmacological and behavioral treatment of cocaine dependence: controlled studies. *Journal of Clinical Psychiatry*, 49, 17-22.

Oppenheim, R.W. (1982). The neuroembryological study of behavior: Progress, Problems, Perspectives, in Hunt R. K. (ed): Current Topics in Developmental Biology, 17, Neural Development, Part III. New York, Academic Press, 1982, 257-309.

Pasternak, G.W. (1988). Multiple morphine and enkephalin receptors and the relief of pain. *J.A.M.A.*, 259, 1362-1367.

Paxinos, G., & Watson, C. (1986). The rat brain in stereotaxic coordinates, Academic Press, San Diego.

Pert, C.B., & Synder, S.H. (1973). Properties of opiate-receptor binding in rat brain. *Proceedings of the National Academy of Science*, USA, 70, 2243-47.

Petty, G.E. (1912). Congenital morphinism with report of cases: general treatment of morphinism. *Memphis M. Montly*, 32, 37-63.

Petty, G.E. (1913). Narcotic Drug Diseases and Allied Ailments, J.A. Davis Co., Tennessee.

Pilcher, C.W.T., & Stolerman, I.P. (1976). Conditioned flavor aversions for assessing precipitated withdrawal morphine abstinence in rats. *Pharmacology, Biochemistry, and Behavior*, 4, 159-163.

Rasmussen, K., Beitner-Johnson, D.B., Krystal, J.H., Aghajanian, G.K., & Nestler, E.J. (1990). Opiate withdrawal and the rat locus coeruleus: Behavioral, Electrophysiological, and biochemical correlates.

Ricalde, A., & Hammer, R. P. (1990). Perinatal opiate treatment delays growth of cortical dendrites. *Neurscience Letters, 115*, 137-143.

Richardson, B., Hohimer, A.R., Mueggler, P., & Bissonette, J. (1982). Effects of glucose concentration on fetal breathing movements and electrocortical activity in fetal lambs. *American Journal of Obstetrics and Gynecology*, 142, 678-683.

Rubino, T., Parenti, M., Patrini, G., Massi, P., & Parolaro, D. (1995). Morphine withdrawal syndrome and G protein expression: a study of the time course in the rat central nervous system. *European Journal of Neuroscience, 7*, 2334-2340.

Schaefer, G.J., & Michael, R.P. (1983). Morphine withdrawal produces differential effects on the rate of lever-pressing for brain stimulation in the hypothalamus and midbrain in rats. *Pharmacology, Biochemistry, & Behavior*, 18, 571-577.

Schroeder, R.L., Weinger, M.B., Vakassian, L., & Koob, G.F. (1991). Methylnaloxonium diffuses out of the brain more slowly than naloxone after direct intracerebral injection. *Neuroscience Letters*, 12, 173-177.

Sharpe, L.G., Garnett, J.E., & Cicero, T.J. (1974). Analgesia and hyperactivity produced by intracranial microinjections of morphine into the periaqueductal gray matter of the rat. *Behavioral Biology*, 11, 303-313.

Sherwood, N.M, & Timiras, P.S. (1970). A stereotaxic atlas of the developing rat brain. University of California Press, Berkley.

Simon, E.G., Hiller, J.M., Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic [3H] etorphine to rat-brain homogenate. *Proceedings of the National Academy of Sciences*, USA, 70, 1947-1949.

Slotkin, T.A., & Seidler, F. (1988a). Adrenomedullary catecholamine release in the fetus and newborn: secretory mechanisms and their role in stress and survival, *Journal of Developmental Physiology*, 10, 1-16.

Slotkin, T.A., & Seidler, F. (1988b). Stress in the fetus and newborn. *Advances in Experimental Medical Biology*, 245, 283-94.

Smith, A. A., Hui, F.W., & Crawford, M. J. (1973). Inhibition of growth in young mice treated with d, l-methadone. *European Journal of Pharmacology, 43,* 307-314.

Smotherman, W. P., Bell, R. W., Starzec, J., Elias, J., & Zachman, T. A. (1974). Maternal responses to infant vocalizations and olfactory cues in rats. *Behavioral Biology, 12,* 55-66.

Smotherman, W. P., & Robinson, S. R. (1986). Environmental determinants of behavior in the rat fetus. *Animal Behavior, 34,* 1859-1873.

Smotherman, W. P., & Robinson, S. R. (1987). Prenatal influences on development: behavior is not a trivial aspect of fetal life. *Developmental & Behavioral Pediatrics, 8,* 171-176.

Smotherman, W. P., & Robinson, S. R., (1992). Kappa opioid mediation of fetal responses to milk. *Behavioral Neuroscience, 106,* 396-407.

Sofuoglu, M., Portoghese, P.S., & Takemori, A.E. (1992). Maintenance of acute morphine tolerance in mice by selective blackage of κ-opioid receptors with norbinaltorphimine. *European Journal of Pharmacology*, 210, 159-162.

Spain, J.W., Roth, B.L., & Coscia, C.J. (1985). Differential ontogeny of multiple opioid (μ,σ, and κ). *Journal of Neuroscience*, 5, 584-588.

Stinus, L., Le Moal, M., & Koob, G.F. (1990). Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. *Neuroscience*, 37, 767-773.

Szeto, H. (1995). Opioid receptor approaches for the development of medications for pregnant women. *NIDA Research Monographs*, 149, 100-115.

Tao, P.L., Lee, H.Y., Chang, L.R., & Loh, H.H. (1990). Urinary catecholamines, plasma activity and blood pressure in newborns: Effects of narcotic withdrawal. *Developmental Pharamacology and Therapeutics*, 3, 83-87.

Taylor, J.R., Elsworth, J.D., Garcia, E.J., Grant, S.J., Roth, R.H., & Redmond, D.E., Jr. (1988). Clonidine infusion into the locus coeruleus attenuates behavioral and neurochemical changes associated with naloxone-precipitated withdrawal. *Psychopharmacology*, 96, 121-134.

Tempel, A., Habas, J., Paredes, W., & Barr, G.A. (1988). Morphine-induced downregulation of μ-opioid receptors in neonatal rat brain. *Developmental Brain Research, 41*, 129-133.

Tempel, A., & Zukin, R.S. (1987). Neuroanatomical patterns of the μ, σ, & κ opioid receptors of rat brain as determined by quantitative in vitro autoradiography. *Proceedings of the National Academy of Science, USA*, 84, 4308-4312.

Tempel, A. (1991). Visualization of μ opiate receptor downregulation following morphine treatment in neonatal rat brain. *Developmental Brain Research, 64*, 19-26.

Terenius, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacology & Toxicology*, 32, 317-320.

Thompson, R.C., Mansour, A., Akil, H., & Watson, S. J. (1993). Cloning and pharmacological characterization of a rat μ opioid receptor. *Neuron*, 11, 903-913.

Tremblay, E.C. & Charton, G. (1981). Anatomical correlates of morphine-withdrawal syndrome: Differential participation of structures located within the limbic system and striatum. *Neuroscience Letters*, 23, 137-142.

Trujillo, K., & Akil, H. (1990). Pharmacological regulation of striatal prodynorphin peptides. *Progress in Clinical. Biology Research.*, 328, 223-226.

Trujillo, K., & Akil, H. (1991). Opiate tolerance & dependence: recent findings & synthesis. *The New Biologist, 3*, 915-923.

Umans, J.G., & Szeto, H.(1983). Effects of opiates on fetal behavioral activity in utero. *Life Science*, 33, 639-642.

Van Kleek, L.A. (1920). Symptoms of morphine withdrawal in an infant. *Am. Med.*, 15, 51-52.

Vivian, J. A., & Miczek, K. A. (1991). Ultrsounds during morphine withdrawal in rats. *Psychopharmacology, 104*, 187-193.

Wallach, R.C., Jerez, E., & Blinick, G. (1969). Pregnancy and menstrual function in narcotics addicts treated with methadone. *Am. J. Obstet.Gynecol.*, 105, 1226-1229.

Wang, A., & Lovick, T.A. (1993). The inhibitory effect of the ventrolateral periaqueductal gray matter on neurons in the rostral ventrolateral medulla involves a relay in the medullary raphe nuclei. *Experimental Brain Research*, 94, 295-300.

Wei, E., Tseng, L. F., Hoh, H., & Way, E. L. (1974). Brain sites of precipitated abstinence in morphine-dependent rats. *Journal of Pharmacology and Experimental Therapeutics, 185*, 108-115.

Wilson, J.C., & Eshner, A.A., (1896). American Textbook of Applied Therapeutics. W.B. Saunders, New York.

Windh, R. T., Little, P. J., & Kuhn, C. (1995). The ontogeny of Mu opiate tolerance and dependence in the rat: antinociceptive and biochemical studies. *Journal of Pharmacology and Experimental Therapeutics, 273, 3,* 1361-1374.

Wooten, G.F. (1993). Functional anatomy of opiate withdrawal. From: The neurobiology of opiates, CRC Press, Inc., Ch. 7, 165-174.

Yasuda, K., Raynor, K., Kong, H., Breder, C.D., Takeda, J., Reisine, T., & Bell, G.I. (1993). Cloning and functional comparison of κ and σ opioid receptors from mouse brain. *Proc. Natl. Acad. Sci. USA*, 90, 6736-6740.

Yoburn, B. C., Chen, J., Huang, T., & Inturrisi, E. (1985). Pharmacokinetics and pharmacodynamics of subcutaneous morphine pellets in the rat. *The Journal of Pharmacology and Experimental Therapeutics, 235*, 282-286.

Yukhananov, R.Y, Klodt, P.M., Il'lna, A.D., Zaitsev, S.V., & Maisky, A.I. (1994). Opiate withdrawal intensity correlates with the presence of DSLET high-affinity binding. *Pharmacology, Biochemistry, and Behavior*, 49, 1109-1112.

Zadina, J. E., Kastin, A. J., Coy, D. H., & Adinoff, B. A. (1985). Developmental, behavioral, and opiate receptor changes after prenatal or B-endorphine, CRF, or Tyr-MIF-1. *Psychoneuroendocrinology, 10*, 367-383.

Zelson, C., Lee, S.T., & Casalino, M. (1973). Neonatal narcotic addiction-10-year observation. *Pediatrics*, 18, 178-189.

Zmitrovich, A., Hutchings, D. E., & Barr, G. A. (1993). Can neonatal abstinence be demonstrated in the seven-day-old rat? Collge on Problems of Drug Dependence, Toronto, (1993).