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THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE STRESS AXIS AND NEUROBEHAVIOR

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THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE STRESS AXIS AND NEUROBEHAVIOR

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Sandra J. Legan, Professor of Physiology

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2017

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ABSTRACT OF DISSERTATION

THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE STRESS AXIS AND NEUROBEHAVIOR

Opiate addiction is now a major public health problem. Pregnant women continue to use opiates during gestation; up to 5.4% of pregnant women report using illicit drugs during pregnancy. Previous studies have shown that perinatal insults and exposure to opiates such as morphine in utero can affect the development of the hypothalamic-pituitary-adrenal (HPA)-axis of the offspring and are associated with higher risk of developing neurobehavioral problems. Oxycodone, a semisynthetic putative kappa opioid receptor and partial mu opioid receptor agonist is now one of the most frequently abused pain killers during pregnancy, however limited data are available regarding whether and how perinatal oxycodone exposure (POE) alters the development and functions of the HPA-axis, the related stress axis and neurobehavioral outcomes of the offspring. Data from these experiments have provided novel evidence that POE indeed is associated with sex-specific changes in the HPA-axis in response to stress that persist beyond the neonatal period. 1) POE is associated with an increased adrenocorticotropic hormone (ACTH) response to corticotropin-releasing hormone (CRH), but not the corticosterone (CORT) response to CRH stimulation in late adolescent male offspring. 2) POE is associated with increased CORT, but not ACTH response to restraint stress test in adult female offspring. These changes in the HPA-axis response to stress may be partially explained by 1) an increase in the subpopulation of CRH neurons that also contain estrogen receptor-beta immunoreactivity following POE which then can exaggerate the stimulation of the HPA-axis, and 2) a decrease in mineralocorticoid receptor-mRNA expression in the hippocampus which may be associated with impaired
negative feedback control of the HPA-axis by the limbic system. POE is also associated with cardiovascular changes in response to stress during a classical conditioning paradigm; adolescent male POE rats have a larger blood pressure increase than the control group. Although POE male rats can properly discriminate the stress versus non-stress cues in the conditioning paradigm, they do not retain this memory when retested during adulthood. When tested for learning and memory in a water maze, however, we did not find any differences between control rats and rats exposed to high dose oxycodone in utero. In addition, we demonstrated that exposure to the lower dose of oxycodone in utero is associated with hyperactivity in adult rats when tested in an open field. Our results make a significant contribution to the literature because they extend our knowledge about the effects of oxycodone on the developing brain and the resulting outcomes in animal models that are actually relevant to a current major public health problem in humans and will provide a platform for us to further study the underlying mechanisms and interventions that may mitigate these effects.

KEYWORDS: Prenatal Opiate Exposure, Prenatal Oxycodone Exposure, Stress Axis, HPA-axis, Cardiovascular, Neurobehavior

Thitinart Sithisarn

05/03/2017

Date
THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE STRESS AXIS AND NEUROBEHAVIOR

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DEDICATION

This work is dedicated to
Pranee Sithisarn, PharmD, MS
My Beloved Mother
This dissertation benefited from the insights and direction of several people. First, my Dissertation Chair and adviser, Dr. Sandra Legan, who exemplifies the high quality scholarship to which I aspire and the technical support from her laboratory, Xiao Li Peng, Jonathan England, Christopher Rhinehardt, DVM and Lindsey Hornung. Next, I wish to thank my committee members, Dr. David Randall and his colleagues (Dr. David Brown and Dennis Silcox), Dr. Susan Barron and her trainee (Dr. Kristen Wellmann), and Dr. Melinda Wilson and her associates (Dr. Jenny Westberry and Dr. Tomoko Sengoku) for their collaboration, support with the experiments and critical comments. I would also like to thank another Dissertation Committee member, and the outside reader, respectively: Dr. Ok-Kyong Park-Sarge and Dr. Octavio Gonzalez. Each individual provided insights that guided and challenged my thinking, substantially improving the finished product. In addition to the technical and instrumental assistance above, I received equally important assistance from family and friends. My husband, Dr. Jozsef Stork provided on-going support throughout the dissertation process. My mother, Pranee Sithisarn, PharmD, MS, who instilled in me, from an early age, the desire and skills to obtain the Ph.D. Finally, I wish to thank Dr. Henrietta Bada, my career mentor for her support and advice.
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CHAPTER ONE
THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE STRESS AXIS
AND NEUROBEHAVIOR
GENERAL INTRODUCTION

Opioid is a broad term for a chemical substance that acts on opioid receptors for pain relief. Opiates, as a group, encompass opioid drugs, many of which are derived from opium poppy plant alkaloid compounds; however, the terms opiate and opioid are often used interchangeably. The prevalence of opiates and prescription opioid abuse among pregnant women has increased dramatically and continues to be a major public health concern. According to the recent data from the National Survey on Drug Use and Health, an estimated 4.3 million people aged 12 or older were nonmedical users of opiate pain relievers in the United States in 2014 (SAMSHA 2014). Up to 5.4% of pregnant women report using illicit drugs during pregnancy, with a higher rate of 14.6% among pregnant teenagers (SAMSHA 2013). Nonprescription opioids are the second most abused illicit substance, second to only marijuana (Wendell 2013). Substance use problems pose socioeconomic burdens on society; workplace, health care, and judicial expenses of the opioid epidemic are estimated to be greater than 50 billion dollars annually (Kremer and Arora 2015). Importantly, substance use in pregnant women and subsequent fetal exposure to drugs has been linked to adverse health effects for the maternal-fetal dyad. Although opioids have been classified as non-teratogenic, the deleterious effects of opioid abuse in pregnant women are associated with the effects of withdrawal for the woman, her fetus and other concomitant risky behaviors (ACOG 2012). In addition, substance abuse, including abuse of opiates, can affect the developing fetus directly or indirectly through various mechanisms. Opiates can cross the placenta (Gerdin, Rane et al. 1990, Nanovskaya, Deshmukh et al. 2002, ...
Nanovskaya, Nekhayeva et al. 2008) and act directly on opioid receptors of the fetus. Opiates such as morphine rapidly cross the placenta with drug equilibration between the mother and the fetus (Gerdin, Rane et al. 1990) while methadone decreases placental permeability, which in turn leads to a decrease in oxygen and nutrient supply from maternal circulation to the fetus (Malek, Obrist et al. 2009). Opiates can also affect maternal physiologic status by enhancing the secretion of cortisol in the mother or stimulating the secretion of stress hormones in the fetus (Taylor, Soong et al. 1997) which can pose long term effects to the developing fetus (Brunton 2015). In addition, maternal rearing behaviors and mother-offspring interactions, which can be negatively impacted by perinatal opiate exposure (Bridges and Grimm 1982, Slamberova, Bar et al. 2003), have been well-described to affect long term neuro-development of the offspring (Sng and Meaney 2009). Therefore, it is important to study the effects of perinatal opiate exposure on the developing brain due in part to these various mechanisms. Moreover, understanding the effects and underlying mechanisms whereby perinatal opiate exposure modulates the developing process of the brain and the stress axis will facilitate finding the approach to manage opioid-addicted mothers and their infants, which in turn may alleviate the negative impacts of perinatal opiate exposure on the offspring.

1.1 The Effects of Perinatal Opiate Exposure on the Developing Brain

Studies in humans show that perinatal exposure to opiates is associated with deleterious effects in the infants, including premature birth, low birth weight (Binder and Vavrinkova 2008) and smaller head circumference (Hunt, Tzioumi et al. 2008). Infants exposed to opiates in utero have high-pitched hyperphonated cries, reflecting a compromised reactivity and neural control of the cry sound (Lester, Tronick et al. 2002). In children, in utero opiate exposure is associated with abnormal electroencephalogram (EEG) findings (van Baar, Fleury et al. 1989), as well as smaller intracranial and regional
brain volumes on magnetic resonant imaging (MRI) with correlating cognitive and behavioral difficulties (Walhovd, Moe et al. 2007). In utero opiate exposure is also associated with a number of neuropsychological and behavioral abnormalities in infants and children. One study shows lower cognitive function at 18 months of age persisting on repeat measurement at age 3 years (Hunt, Tzioumi et al. 2008). Children exposed to opiates score lower than non-exposed controls in the Wechsler Intelligence Scale-Revised (Davis and Templer 1988). An increased rate of attention deficit hyperactivity disorder is found in children born to opiate-addicted parents (Ornoy, Segal et al. 2001). Importantly, disruption of opioid transfer through the placenta after delivery leads to the development of Neonatal Abstinence Syndrome (NAS). NAS typically manifests with both central and autonomic nervous system signs and symptoms, such as muscle hypertonicity, hyperreflexia, hyperactivity, irritation, gastrointestinal dysfunction and seizures (Bada, Bauer et al. 2002, Kocherlakota 2014). Even though the incidence of NAS has increased dramatically in recent years due to an epidemic use of opiates during pregnancy, the long-term consequences on cognitive function and neurobehavior remain unclear and require further study. Moreover, research in this area is still in formative stages with limitations that make it difficult to define mechanistic effects of perinatal opiate exposure. These include maternal poly-substance use, postnatal environment and other stressors, variations in timing, route and dosage of administration. Very limited data are available on the specific effects of oxycodone on the developing fetus.

The animal models used to study the effects of perinatal drug exposure provide better control of the confounders, timing, dosage and route of administration and allow a mechanistic approach. Animal studies showed that prenatal opiate exposure had both short and long-term effects on offspring. Similar to human clinical studies, rodents
exposed to opiates (morphine and heroin) had a lower birthweight (Eriksson and Ronnback 1989, Lu, Liu et al. 2012). Long term effects of perinatal opiate exposure include impaired learning and memory (Slamberova, Schindler et al. 2001, Schrott, Franklin et al. 2008, Davis, Franklin et al. 2010), impaired locomotor behaviors and drug susceptibility (Wong, Lee et al. 2014, Tan, Duan et al. 2015). Prenatal morphine exposure suppresses the stress response of the HPA-axis (Vathy 2002, Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004, Hamilton, Harris et al. 2005). Structural and functional alterations in animal models may underlie the observed behavioral changes and impairment in learning and memory and the dysregulation of the HPA-axis; these include perturbations in dendritic length in the somatosensory cortex (Lu, Liu et al. 2012), synaptic plasticity in the dentate gyrus and hippocampal CA1 and CA3 region (Yang, Liu et al. 2006, Villarreal, Derrick et al. 2008), neuronal proliferation and cell survival (Oliveira, Rego et al. 2002, Svensson, Bucht et al. 2008, Wang and Han 2009), and cholinergic function (Steingart, Abu-Roumi et al. 2000). Most of these animal studies used morphine, heroin, methadone or other opiates such as l-alpha-acetylmethadol (LAAM) in the models, with very little data available on the effects of perinatal oxycodone exposure on the long-term outcomes. Because of the higher incidence of pain reliever abuse, and because different opiate agonists can affect the developing fetal nervous system differently (as described below), we were interested specifically in studying the effects of perinatal oxycodone exposure (POE) on the neurodevelopment and stress axis of the offspring. How different opioids, such as morphine versus oxycodone, affect the stress system differently, may be in part due to the different opioid receptor subtypes they act on.
1.2 Opioid Receptors, Opioid Receptor Agonists and How Oxycodone Can Pose Different Effects Compared to Other Previously Studied Opiates.

Opioid is the term used to describe all compounds that work at the opioid receptors throughout the tissues (Trescot, Datta et al. 2008). Opioid receptors (ORs) belong to the super-family of G-protein coupled receptor (GPCRs) which are the most abundant class of cell-surface receptors (Shang and Filizola 2015). Multiple opioid receptor subtypes have been identified from the anatomical location and pharmacological profiles of compounds that were eventually used to name them. There are three major subtypes of opioid receptors: delta (δ) receptor (DOR), mu (μ) receptor (MOR) and kappa (κ) receptor (KOR) (Dietis, Rowbotham et al. 2011). The fourth receptor subtype, the nociception/orphanin opioid receptor is phylogenetically related to other opioid receptors but does not bind the same ligands (Dietis, Rowbotham et al. 2011). These receptors can be activated by endogenous peptides such as enkephalins, endorphins and dynorphins, but are also activated by natural alkaloids such as morphine and codeine, semi-synthetic opioids, which are a combination of natural opioids and synthetics such as oxycodone, and synthetic opioid compounds such as fentanyl (Trescot, Datta et al. 2008). Besides analgesic effects, the actions of opioids on opioid receptors have a wide range of vital functions, including stress regulation, reward, learning/memory and immunological responses. The activation of different subtypes of ORs leads to different molecular-biochemical and physiological effects (Bodnar 2014). ORs are expressed at high levels in multiple brain areas and cell types during early embryonic development in both rodents (Kornblum, Hurlbut et al. 1987) and humans (Magnan and Tiberi 1989, Wang, Dow-Edwards et al. 2006, Tripathi, Khurshid et al. 2008). Opioid systems play diverse roles in regulating neural cell growth, differentiation (Hauser and Mangoura 1998) and dendritic growth and spine formation (Hauser,
McLaughlin et al. 1987). More recent studies also show that opioid systems can contribute to many important aspects of brain development, including inhibition of neuronal proliferation and differentiation (Hauser, Houdi et al. 2000), neurite extension (Tsai, Tsui et al. 2010) and the effects of astrocytes on brain plasticity (Slezak, Korostynski et al. 2013).

Morphine, the commonly considered archetypal opioid analgesic, activates mainly the MOR (Trescot, Datta et al. 2008). On the other hand, oxycodone is a putative KOR agonist but also activates MOR (Horan, de Costa et al. 1991, Ross and Smith 1997). Nielsen et al show that oxycodone and morphine have distinctly different pharmacological profiles and produce antinociception through distinct ORs; there is an absence of antinociceptive cross tolerance to intracerebroventricular oxycodone treatment in rats tolerant to morphine. Oxycodone appears to act as a Kappa (2b)-opioid agonist with a relatively low affinity for mu-opioid receptors (Nielsen, Ross et al. 2007). Both MOR and KOR receptors are expressed throughout the brain but with distinct neuro-anatomical distributions (Sharif and Hughes 1989). Although the acute administration of either MOR or KOR agonists produces analgesic effects (Benyhe 1994, Chartoff and Mavrikaki 2015) and stimulates the response of the HPA-axis, at both ACTH (Houshyar, Gomez et al. 2003, Szeto 2003) and glucocorticoid levels (Taylor, Soong et al. 1997) perinatal exposure to MOR or KOR agonist may have different long term effects on the regulation of motivational processes, including the modulation of the stress axis as will be discussed in more detail below and in later chapters.

1.3 HPA-axis System Review and Interaction of the Stress System with Opioids

Adaptation and maintenance of homeostasis when challenged with stress require the coordinated activation of the neuroendocrine systems which include the
hypothalamic pituitary adrenal axis (HPA-axis), sympathetic nervous system, immune system and behavioral responses. The stimulation of the HPA-axis results in increases in circulating glucocorticoids, the major endocrine response to stress (de Kloet, Joels et al. 2005). The cascade starts with the stimulation of the neurons in the dorsomedial parvocellular subdivision of the paraventricular nucleus (PVN) of the hypothalamus. These neurons synthesize and release corticotropin-releasing hormone (CRH) as well as vasopressin (VP) to the pituitary portal circulation (Swanson, Sawchenko et al. 1983, Sawchenko, Swanson et al. 1984). The binding of CRH to type 1 CRH receptors (CRHR1) and VP to V1b receptors in the anterior pituitary corticotropes stimulates increased synthesis of the prohormone proopiomelanocortin (POMC) and release of its cleavage products, including ACTH (Adrenocorticotropic hormone), γ-lipotropin and β-endorphin, into the peripheral circulation (Vale, Rivier et al. 1983, Antoni 1986, de Kloet, Joels et al. 2005). ACTH stimulates adrenal glucocorticoid secretion which is vital for the metabolic adaptation and modulation of brain function in response to stress (Aguilera 2012). Glucocorticoids also exert feedback inhibition of the HPA-axis at the pituitary (Drouin, Charron et al. 1987), the PVN and the limbic structures (Herman, Ostrander et al. 2005, Herman, McKlveen et al. 2016) through glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (more details in section 1.5.2). CRH also modifies behavioral and autonomic adaptation of the brain by binding to CRHR1 outside the pituitary in the limbic structures (Aguilera 2012). The limbic structures which include the hippocampus, the amygdala, and the prefrontal cortex (PFC) provide counter balance, i.e., both negative and positive feedback control of the HPA-axis as will be described below.

The complex neurocircuitry between the limbic structures and the PVN provides control of the HPA-axis response to stress. The regulatory functions of the limbic
structures on the PVN are likely stimulus-specific and heterogeneous based on the anatomical micro-organization of each structure (Jankord and Herman 2008). The hippocampus exerts primarily an inhibitory, trans-synaptic influence on the PVN as demonstrated by elevated basal glucocorticoid levels and disruptions of the diurnal rhythm after hippocampal lesions (Fendler, Karmos et al. 1961, Fischette, Komisaruk et al. 1980, Jankord and Herman 2008). Further, the HPA activity (corticosterone level) is inhibited by the stimulation of the ventral hippocampus (Casady and Taylor 1976). On the other hand, the electrical stimulation of the amygdala increases corticosteroid secretion both in rats (Redgate and Fahringer 1973) and humans (Gallagher, Flanigin et al. 1987), suggesting its stimulatory controlling role on the PVN. With limited projections to the PVN, the amygdala likely affects PVN neurons via indirect pathways by reducing the inhibitory inputs to the PVN (Herman, Figueiredo et al. 2003). The PFC is involved in the stress response by processing stressful information and interacts with other sites to communicate the inhibitory signals of the HPA axis activity (Jankord and Herman 2008). Robust c-fos induction is observed in the PFC following multiple stress paradigms (Duncan, Johnson et al. 1993, Cullinan, Herman et al. 1995) and region-specific lesions of the PFC enhance ACTH and corticosterone in response to stress (Figueiredo, Bruestle et al. 2003). Therefore, if there are alterations in the function of the HPA-axis in response to stress, they could be the result of changes in the regulatory inputs from the limbic structures. We then may hypothesize that prenatal exposure to oxycodone may be associated with a decrease in inhibitory input from the hippocampus and PFC, and/or an increase in the stimulatory control from the amygdala which then results in an enhanced response to stress of the PVN.
1.3.1 Modulation of the HPA-Axis by the Opioid System

The HPA-axis activity can be modulated at different levels along the axis and also by interactions with not only the limbic structures as mentioned, but also the endogenous opioid system. The endogenous opioid system acts to oppose the effects of the major stress neuromodulator, CRH, through the ORs, and to promote the recovery from stress (Drolet, Dumont et al. 2001, Valentino and Van Bockstaele 2015). Likewise, exogenous opioids can also modulate the HPA-axis via actions on ORs as will be described below (Pechnick 1993).

There are three independent genes that are transcribed to three precursor proteins, which give rise to the endogenous opioid peptide families: proopiomelanocortin (POMC), preproenkephalin (PENK) and preprodynorphin (Benarroch 2012). The active peptides endorphin, enkephalin and dynorphin bind to μ-, δ- and κ-receptors respectively. These endogenous opioids bind to opioid receptors that are widely distributed throughout the HPA-axis, i.e., the hypothalamus, pituitary and adrenal gland (Goldstein and Ghazarossian 1980). Endogenous opioids are released by stressors and modulate the stress response (Valentino and Van Bockstaele 2015). This is supported by data from animal studies indicating that stressors, whether they are noxious or not, produce analgesic effects that are cross-tolerant with morphine and antagonized by naloxone (Lewis, Cannon et al. 1980, Miczek, Thompson et al. 1982, Rodgers and Randall 1985). Endogenous opioid systems may modify the stress response through multiple mechanisms. Dynorphin is the endogenous ligand of the kappa-opioid receptor. Dynorphin-like peptides have been found to co-localize with CRH. These peptides activate the hypothalamic release of CRH and arginine vasopressin via opioid and non-opioid-dependent mechanisms, thereby activating ACTH release in sheep (Szeto 2003).
Therefore it is possible that oxycodone which can stimulate KOR may activate the HPA-axis through the same mechanisms as these dynorphin-like peptides.

1.3.2 Modulation of the HPA-Axis by Exogenous Opioids/ Opiates

Exogenous opiates also modulate the HPA-axis in opioid receptor (whether KOR or MOR agonists)-, dose- and species-specific manners. However, even in the studies using morphine, the results are still conflicting. Differences in the results are likely due to exposure paradigms. The modulation of the HPA-axis by prenatal exposure to opiates such as morphine or oxycodone will be discussed in the following section. Studies show that an acute morphine challenge has stimulatory effects on the HPA-axis in rats and sheep (Taylor, Soong et al. 1997, Houshyar, Gomez et al. 2003). Nevertheless, more recent animal studies show that short-acting MOR agonists such as morphine and heroin reduce stress-induced HPA-axis activity as counter-regulatory modulators (Zhou and Leri 2016). In contrast, the exogenous KOR specific agonist MR-2034 stimulates the HPA axis in rats by acting at both the hypothalamic and the pituitary levels in a dose-dependent manner (Calogero, Scaccianoce et al. 1996). In addition, chronic opioid exposure can potentially interfere with CRF regulation and the HPA-axis in a receptor-specific manner (Ignar and Kuhn 1990, Valentino and Van Bockstaele 2015). Chronic morphine exposure in adult rats sensitizes one of the brain’s norepinephrine systems, originating in the locus coeruleus, to CRF and physiological stress (Xu, Van Bockstaele et al. 2004). Chronic exposure to a KOR agonist alters the HPA-axis differently compared to MOR agonists. For example, spontaneous and antagonist-precipitated withdrawal from morphine, but not U50,488 (KOR agonist), resulted in elevation of corticosterone levels. In addition, morphine suppressed morphine abstinence-induced corticosterone hypersecretion, whereas, U50,488 had no effect (Ignar and Kuhn 1990, Milanes, Gonzalvez et al. 1991). However, how chronic oxycodone exposure modulates
the HPA-axis during the developmental period of the HPA-axis and stress system has not been studied. Therefore, the work from this dissertation will fill this gap in knowledge.

1.3.3 The Effects of Perinatal Opiates or Stress Exposure on the HPA-axis

In many mammalian species, the HPA-axis is well developed and functional in late gestation (Matthews and Challis 1996). However, in rats the development of the HPA-axis is not completed until postnatal day 5-7, therefore the late pre- and early postnatal periods are critical for the development of the HPA-axis (Gutman and Nemeroff 2002). Exposure to stressors during this vulnerable period can lead to life-long changes in the HPA-axis and stress response (Silberman, Acosta et al. 2016). The effects of prenatal/early postnatal stress exposure have been well studied in both animal and human models. Early life exposure to stress impairs the regulation of the HPA-axis, increases anxiety behaviors and leads to cognitive impairment in the offspring (Weinstock 1997, Brunton 2015).

The effects of prenatal exposure to opiates on the regulation and function of the HPA-axis have also been studied, but the results are mixed, and so far, the focus has been on the exposure to morphine. Although the dose and route of the morphine administration in these studies are the same, differences in the results are likely due to the experimental designs, namely whether the offspring are challenged with stress. In the study by Rimanoczy et al, prenatal morphine exposure suppresses the restraint stress test (RST) induced ACTH response with no changes in the corticosterone (CORT) response in both male and female offspring (Rimanoczy, Slamberova et al. 2003, Slamrova, Rimanoczy et al. 2004). On the other hand, a different study by Dutriez-Casteloot et al did not find significant effects of prenatal morphine exposure on the HPA-axis; under resting conditions, prenatal morphine exposure did not change the activity of
the HPA-axis, including hypothalamic CRF content, plasma ACTH concentrations, adrenal weight and CORT content and plasma CORT level in adult male offspring (Dutriez-Casteloot, Bernet et al. 1999). The probable reason why the latter study did not detect the effects of prenatal morphine exposure on the HPA-axis could be that the study was done under resting, not during stress conditions. Therefore, prenatal morphine exposure likely has suppressive effects on the HPA-axis response to stress based on the two former studies. However, it is not known how perinatal exposure to oxycodone impacts the development and functions of the HPA-axis that may persist to adulthood. Therefore, we proposed to study the effects of perinatal oxycodone exposure on the HPA-axis response to stress in the offspring, as outlined in Chapter 3.

1.4 Working Hypothesis for the Effect of Perinatal Oxycodone Exposure on the HPA-axis

Prenatal morphine exposure has suppressive effects on the HPA-axis based on the studies mentioned in section 1.3.3 (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). But unlike morphine, oxycodone acts mainly on kappa opioid receptors which may cause different effects on the developing brain. Therefore we hypothesize that perinatal oxycodone exposure will affect the HPA-axis development which will be manifested as an enhanced HPA-axis response to stress. We proposed to test this hypothesis using a CRH stimulation test, a direct pharmacological challenge to the HPA-axis, as will be described in Chapter 3. In addition, we will test this hypothesis using a restraint stress test, one of the most commonly used stress tests to induce classical stress-related behavioral, biochemical and physiological responses in laboratory animals, as will be described in Chapter 4.
1.5 Proposed Mechanisms by Which Perinatal Oxycodone Exposure Affects the Stress Axis

Limited data are available in regard to the mechanisms by which perinatal opiate exposure alters the function and regulation of the HPA-axis. We speculate that perinatal oxycodone exposure may alter 1) the expression of the CRH in the hypothalamus and limbic structures, and 2) the feedback mechanisms of the HPA-axis via the actions of glucocorticoid on glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in the PVN (see below) and the counter balance control of the HPA-axis by the limbic system.

1.5.1 CRH neurons

Previous studies show that adverse prenatal environments such as undernutrition or chronic fetal stress impact CRH expression in the stress axis (Nunez, Ruiz et al. 2008, Garcia-Caceres, Lagunas et al. 2010). For example, CRH mRNA expression determined by in situ hybridization in the central nucleus of the amygdala is increased in male mice exposed to prenatal stress (Mueller and Bale 2008). CRH-receptor mRNA expression patterns in the amygdala are increased in a sex-specific manner, only in the male, in rats prenatally exposed to stress (Brunton, Donadio et al. 2011). Thus far there is no study on the effect of perinatal opiate exposure on CRH mRNA and protein expression. Since we hypothesize that POE may enhance the HPA-axis to stress, we then further hypothesize that POE will increase CRH mRNA and protein expression in the PVN and limbic structures and that this increase may be sex-specific; may occur only in the males. This hypothesis will be tested in Chapter 5.

1.5.2 Feedback Mechanisms of the HPA-axis

Inhibitory feedback by glucocorticoid (cortisol in humans and corticosterone (CORT) in rats) via GR and MR plays a major role in limiting the response of the HPA
axis to stress at the pituitary and central levels (Jacobson and Sapolsky 1991). At the pituitary level, glucocorticoids inhibit both the ACTH secretory mechanism and the synthesis of the ACTH precursor molecule, POMC (pro-opiomelanocortin) (Keller-Wood 2015). Both pituitary and PVN express MR (Spencer, Miller et al. 1993, Han, Ozawa et al. 2005). However, the site of the MR effect on basal glucocorticoid secretion is thought to be at the hippocampal level when hippocampal MRs are significantly occupied (Spencer, Miller et al. 1993).

At the central level, glucocorticoid negative feedback controls the basal ACTH level and activity of CRH neurons via inhibition of CRH mRNA expression (Evans, Liu et al. 2013), CRH release from the PVN, and excitation of the inhibitory hippocampal circuit (Keller-Wood 2015). The hippocampus expresses high levels of both GR and MR. MRs located in the hippocampus have high affinity to glucocorticoids and are sensitive to very low levels of circulating glucocorticoid, therefore MRs are essential to maintain basal activity of the HPA-axis (de Kloet, Otte et al. 2016), while both GR and MR are required at the circadian peak (Dallman, Akana et al. 1987, Bradbury, Akana et al. 1994). The systemic (physical and metabolic) stressors require immediate response from the HPA axis and involve monosynaptic ascending pathways from the brainstem and spinal cord with direct projections to the PVN (Palkovits, Zaborszky et al. 1980, Pacak and Palkovits 2001, Herman, Figueiredo et al. 2003). In contrast, HPA-axis response to psychogenic stressors utilizes complex polysynaptic pathways including several limbic structures (hippocampus, PFC and amygdala) and bed nucleus of the stria terminalis. The hippocampus sends inhibitory signals through the PFC and subiculum; these regions in turn innervate the periventricular region containing glutamatergic and γ-aminobutyric acid (GABA)ergic projections to the CRH neurons (Ziegler, Cullinan et al. 2005). GRs in the limbic structures also play important roles in the feedback mechanism of the HPA-
axis in response to stress. GR has relatively low affinity to glucocorticoid compared to MR (Reul and de Kloet 1985), therefore, is responsible for feedback mechanisms when circulating glucocorticoid levels are in the stress range (Keller-Wood 2015). However, GR over-expression or forebrain-specific GR knockout experiments in mice show that GRs in the forebrain limbic region (prefrontal cortex, hippocampus and amygdala) are essential for maintaining basal glucocorticoid secretion along with MR, and they also play a role in the HPA-axis response to acute psychological stress (Furay, Bruestle et al. 2008).

We are not aware of any available data in regard to the effect of perinatal oxycodone exposure on the feedback mechanisms of the HPA-axis; however, data from perinatal stress studies strongly suggest that perinatal events lead to changes in the HPA-axis feedback mechanisms, which in turn affect the HPA-axis response to stress. Prenatal stress or exposure to glucocorticoid decreases GR and MR expression in the hippocampus, PFC and other feedback sites (Shoener, Baig et al. 2006, Mueller and Bale 2008, Green, Rani et al. 2011, Harris and Seckl 2011) and these changes are sex-specific (Mueller and Bale 2008). Sex-specific changes have also been observed in prenatal morphine exposure models; the stress even from vehicle injections up-regulates the MR and GR binding sites in the hippocampus, of only male rats. These changes in the feedback mechanism are protected, i.e., abolished, by prenatal morphine exposure (Rimanoczy, Slambergerova et al. 2006). The evidence from perinatal stress and morphine exposure models supports our hypothesis that changes in the HPA-axis response to stress after perinatal oxycodone exposure could be explained partly by changes in the feedback mechanisms (GR, MR expression) at the level of hypothalamus and/or limbic structures (hippocampus, amygdala and prefrontal cortex). We propose to test this hypothesis as will be described in Chapter 5.
1.5.3 Gender Differences and the Effects of Estrogen Receptors (ERs) on the HPA-axis

As recently mentioned, there are sex differences in how perinatal exposure to opiates or stress modifies the development of the HPA-axis. Gonadal hormones play an important modulatory role in the function of the HPA-axis (Viau and Meaney 1991, Lund, Munson et al. 2004). In male rats androgen inhibits, whereas in females, estrogens function to enhance the activity of the HPA-axis (Handa, Burgess et al. 1994, Viau and Meaney 1996, Lund, Munson et al. 2004) via actions on estrogen receptor beta (ERbeta) (Miller, Suzuki et al. 2004, Lund, Hinds et al. 2006). High levels of ERbeta are expressed by neurons within the PVN (Suzuki and Handa 2005). Around 12% of medial parvocellular CRH neurons in the PVN contain ERbeta (Miller, Suzuki et al. 2004), while ERalpha expression in the PVN is scarce (Shughrue, Lane et al. 1997). ERbeta mediates the effects of gonadal hormones on the HPA-axis through CRH-dependent mechanisms, in an inhibitory manner for androgen (Lund, Hinds et al. 2006) and a stimulatory manner for estrogens (Miller, Suzuki et al. 2004, Chen, Zhu et al. 2008). ERs are also expressed differentially throughout the brain, including the limbic structures (Laflamme, Nappi et al. 1998, Mitra, Hoskin et al. 2003), and modulate the stress response. For instance, data suggest that the stimulation of ERβ activates an inhibitory circuit within the amygdala and extended amygdala, which then reduces the activation of outputs regulating anxiogenic responses (Handa, Mani et al. 2012). No data are available regarding the effects of perinatal opiates or stress on the expression of the ERs. Therefore we hypothesize that perinatal oxycodone exposure may alter the expression of ERs in the PVN CRH neurons and in the limbic structures, in a sex-specific manner, which may in turn modify the function of the HPA-axis, as will be described in Chapter 5.
1.6 The Interaction of the HPA-axis and other Stress Systems: the Effects of Perinatal Oxycodone Exposure on Cardiovascular Response to Stress (Chapter 6) and Recall Memory

Besides the HPA-axis, the sympathetic-adrenal-medullary (SAM)-axis intimately regulates responses to stress and maintains homeostasis (Carrasco and Van de Kar 2003, de Kloet, Joels et al. 2005). Change in the SAM-axis control is a major contributor to the regulation of blood pressure (Mizuno, Siddique et al. 2013, Grassi and Ram 2016), a major cardiovascular output of the SAM-axis. A growing body of literature suggests that some perinatal insults, such as maternal smoking or perinatal stress, are associated with hypertension of the offspring later in life (Bakker and Jaddoe 2011, Alexander, Dasinger et al. 2015). However, currently there are no studies on the effects of perinatal opiates including oxycodone on either the SAM-axis or cardiovascular response to stress. We then hypothesize that POE will increase the blood pressure response to acute stress during a classical conditioning paradigm, and this was investigated in the studies described in Chapter 6.

In addition, prenatal exposure to opiates is associated with impaired learning/memory (Niu, Cao et al. 2009, Wang and Han 2009, He, Bao et al. 2010). Specifically, male rats prenatally exposed to oxycodone had a greater number of reference memory errors in the radial arm maze, had a modest deficit in retention of the task in the T-maze when assessed 5 days after acquisition training ended, and showed a decreased use of spatial strategies and increase in non-spatial strategies in the Morris water maze. These findings suggested that POE impaired learning and memory in male rats; females were not tested (Davis, Franklin et al. 2010). Therefore, we hypothesize that POE may impair how the offspring differentiate between stress versus non-stress stimuli, which reflects the offspring’s memory, and this hypothesis was tested in the
studies described in Chapter 6. In this chapter we only tested male rat offspring, female offspring were not tested.

1.7 The Interaction of the HPA-axis and Behavior: the Effects of Perinatal Oxycodone Exposure on Behavior (Chapter 7)

Learning and memory problems (Lupien, de Leon et al. 1998, Wirth 2015) and multiple other neuropsychiatric disorders, including anxiety, posttraumatic stress disorder (PTSD) (Shea, Walsh et al. 2005), and depression (de Kloet, Joels et al. 2005) have been linked to the dysregulation of the HPA-axis. It is well supported by previous studies that the effects of adverse perinatal environments on the HPA-axis are associated with behavioral problems and psychopathological traits in the offspring, including anxiety and depression (Mueller and Bale 2008, Van den Hove, Leibold et al. 2014, Grundwald and Brunton 2015). Data from human studies indicate that children exposed to opiates in utero develop hyperactivity, impulsivity, attention problems (Walhovd, Moe et al. 2007, Sundelin Wahlsten and Sarman 2013) and learning problems (Guo, Spencer et al. 1994, Bunikowski, Grimmer et al. 1998, Hunt, Tzioumi et al. 2008). Animal models also show that prenatal exposure to opiates is associated with neurobehavioral problems, such as learning and memory deficits in the offspring (Niu, Cao et al. 2009, Davis, Franklin et al. 2010). However, limited data are available on whether changes in the HPA-axis response to stress after prenatal oxycodone exposure are associated with altered neurobehavioral outcomes in the offspring. Therefore, we hypothesize that POE is associated with detrimental changes in neurobehavioral outcome, including reaction to stress after separation from the mother, hyperactivity and impaired learning and memory, as will be tested and described in Chapter 7. A summary of the HPA-axis, and its feed-back mechanism, its interaction with and modulation by the limbic system and
gonadal hormones, and the interaction with the SAM-axis as another system in the stress response are outlined in Figure 1.
**Figure 1:** Regulation of the HPA –axis stress response (in green) and feed-back mechanism (in red), modulation by the limbic system (in blue) and gonadal hormones (in purple), and interaction with the SAM-axis (in orange); CeA: central nucleus of amygdala, BNST: bed nucleus of the stria terminalis, PFC: prefrontal cortex, NTS: nucleus tractus solitarius, VL: ventrolateral medulla
2.1 Animals

The first set of experiments described in Chapter 3 was performed using timed pregnant Dark Agouti (DA) rats (Harlan, Indianapolis, IN). DA rats were initially chosen for the experiment because as mentioned in Chapter 3, DA rats were reported to be a better model for humans than Sprague-Dawley (SD) rats. However, it was subsequently reported that both SD and DA rats were suitable for use in studies on the effects of oxycodone (Huang, Edwards et al. 2005). Therefore, we chose to use the more available SD rats in all subsequent experiments as described in Chapters 4, 5, 6 and 7. In addition, we began using virgin females which were mated in our animal facility.

All animals were maintained under constantly controlled temperature (22-25 °C) with regulated 30-70% humidity and photoperiod (14L:10D, lights on at 0500 h). Food and water were provided ad libitum.

2.2 Anesthesia

Isoflurane was applied to anesthetize the animals during right atrial cannulation and before brain perfusion.
2.3 Right Atrial Cannulation

This right atrial cannulation procedure was performed on GD 5-6 for the timed pregnant DA rat dams in Chapter 3 and after acclimatization for virgin female SD rats in Chapter 4, 5, 6 and 7. Each rat was fitted with a right atrial cannula under isoflurane anesthesia as previously described with some modification (Mactutus, Herman et al. 1994, Mactutus 1999). The relatively small catheter was hand-crafted from two sizes (internal diameter 0.025 and 0.03 inch) of Silastic medical grade tubing (SF Medical, Hudson, MA). The connection was reinforced with a bead of Silastic adhesive. The tip of the catheter was cut at approximately 45º and located in the right atrium after placement. In the studies in Chapter 3, the distal end of the catheter was tunneled subcutaneously to exit the skin dorsally between the shoulder blades and plugged with a sterile 20 ga blunted needle filled with clay. In all subsequent studies (Chapters 4-7), the distal end of the catheter was similarly tunneled to a dorsal location, but was attached to a sterile Luer-lock injection port that remained in a dorsal subcutaneous pouch thus providing percutaneous access for flushing and injections. The cannulae were flushed with heparinized saline every day to maintain patency and to acclimatize the dam to handling. Beginning on GD 8, each dam was slowly injected over 2 minutes with either oxycodone hydrochloride (Mallinckrodt, St. Louis, MO) 0.8 mg/kg in normal saline solution (NSS) or 1ml/kg NSS once daily via the s.c. port. Heparinized NSS was used to flush the cannula after each injection to keep them patent. Injections continued from GD 8 to 21. On the day of delivery, the pups were counted, weighed, sexed and checked for gross physical abnormalities. The litters were adjusted to 10-12 pups each with equal numbers of males and females when possible. Since the pups' brain development in the first postnatal days corresponds to the human fetal brain development in the third trimester, and withdrawal symptoms may affect maternal nursing behavior, the dams continued to
receive oxycodone or NSS injection on postnatal days (PD) 1, 3, and 5 at the same dosage. The pups were weighed every other day and weaned at PD 25 when they were separated by sex. The dams were euthanized with injection of a euthanizing solution (Beuthanasia-D-special™ Schering-Plough, Union, NJ) through their cannulae.

2.4 Vaginal Smear Cytology

To determine estrous cycle stages of the female rats before mating (Chapter 4, 5, 6 and 7), and to confirm pregnancy, daily vaginal lavage for cytology was performed. Vaginal lavage with clean tap water, volume approximately 0.1-0.2 ml was performed using an eyedropper with a smooth tip. Samples were placed on a gridded 1x3 inch glass slide. Estrous cycle stages (estru, metestrus, diestrus and proestrus) were identified by vaginal smear cell types (leukocytes, nucleated or non-nucleated epithelial cells, cornified cells) under 40X magnification (Cora, Kooistra et al. 2015). In chapter 3, timed pregnant DA rats were used in the experiment. For Chapters 4, 5, 6 and 7, each female was group-housed with a proven breeder male for breeding one week after cannulation. Gestational day 0 was identified when sperm were detected and leukocytic vaginal smears were observed on subsequent days.

2.5 Blood Sample Collection and Plasma Preparation

In Chapter 3, blood samples (0.3 ml) were collected from right atrial cannulae for determination of plasma ACTH and CORT before (0 min baseline) and at 15, 30 and 60 min after CRH injection. To prevent stress from loss of blood volume, 0.3 ml NSS was administered after each sample. Blood samples were collected into tubes containing 1.2 mg ethylenediaminetetraacetic acid (EDTA), and immediately refrigerated (10º C). Once all samples were obtained, the plasma samples were separated by centrifugation and stored at -80º C for future assays. In Chapter 4, blood samples were collected from a tail
At each time point, blood samples (120 µl X 2 tubes, for ACTH and CORT respectively) were collected into microtubes containing 1.2 mg EDTA, and immediately refrigerated (10 º C). Once all samples were obtained, the plasma samples were separated by centrifugation, and stored at - 80° C for future assays.

### 2.6 Brain Perfusion and Brain Sectioning

Rats were deeply anesthetized with isoflurane in the afternoon after they completed RST (Chapter 5). The thorax was opened and 100 units of heparin (0.1 ml of 1000 IU/ml Heparin) were infused through the left ventricle into the aorta followed by 250-300 ml of Dulbecco’s phosphate-buffered saline (DPBS) (Invitrogen, Waltham MA). Perfusate was drained from the right atrium to flush out red blood cells. Then 200-250 ml of 4% buffered paraformaldehyde containing 2.5% acrolein (Sigma-Aldrich, St. Louis, MO) (pH 7.34-7.43) was infused to fix the brain followed by 200-300 ml of DPBS. The brains were removed and saved in 25% sucrose solution. Serial sections of the hypothalamus were cut from the whole brain on a cryostat at a thickness of 20 µm and stored in cryoprotectant. The sections were collected in 10 series of sections 200 microns apart (Watson, Wiegand et al. 1986) that were stored at - 20° C until processed by dual label immunocytochemistry. Selected sections from Bregma -1.4 to Bregma -1.8 (all anatomical references are based on the Paxinos and Watson Atlas (Paxinos 1998)) were processed subsequently by immunocytochemistry.

### 2.7 Radioimmunoassay

In Chapters 3 and 4, both ACTH and CORT levels were determined by radioimmunoassay (RIA) using RIA kits (MP Biomedicals, Orangeburg, NY). The samples were diluted to fit within the linear portion of the standard curves. The CORT kit was designed and validated for use in rat plasma. The ACTH kit was designed for
human plasma samples but we had previously used this product for SD rat plasma samples (Sithisarn, Bada et al. 2011); rat ACTH is biologically and immunologically very similar to human ACTH (Matsuyama, Ruhmann-Wennhold et al. 1970). The sensitivities, based on the lowest dose of standard, were 14 pg/ml and 25 ng/ml for the ACTH and CORT assays, respectively. In Chapter 3, an inter-assay coefficient of variation of was 5.0 ± 1.1 %, and the intra-assay coefficient of variation was 4.4% for the CORT assays (n=2 kits); an inter-assay coefficient of variation of was 6.6 ± 6.6 % and the intra-assay coefficient of variation was 6.8% for the ACTH assays (n=2 kits). In Chapter 4, we performed 7 ACTH assays and 7 CORT assays. The inter-assay coefficient of variation was 3.4 ± 2.8 %, and the intra-assay coefficient of variation was 4.4% for the CORT assays. The inter-assay coefficient of variation was 4.8 ± 1.5 % and the intra-assay coefficient of variations was 6.8% for the ACTH assays.

2.8 Immunocytochemistry (ICC)

The ICC procedure for localization of ERbeta in CRH neurons of the PVN was performed similar to a previously described method (Legan and Tsai 2003). Selected 30 μm sections of the PVN (corresponding to Bregma -1.4 to Bregma -1.8, all anatomical references are based on the Paxinos and Watson Rat Brain Atlas (Paxinos 1998)) were processed as described in detail in Chapter 5. The antibodies were rabbit anti-human ERbeta, dilution 1:1000 (Zymed) and rabbit anti-CRH (courtesy of Dr. Ann-Judith Silverman), dilution 1:50,000. To reduce individual variation in staining, sections from all treatment groups and from both sexes were included in each run of the ICC.
2.9 Analysis of CRH- ERbeta-immunopositive Neurons

All sections were examined under a light microscope (Eclipse E600, Nikon). CRH and ERbeta immunopositive neurons from both left and right hemispheres were identified in the PVN by visualization at magnification of 200-400X. ERbeta immunopositive CRH neurons were counted as positive when a black product was identified in the nucleus and a brown chromogen product could be identified in the cytoplasm of the same cell. ERbeta immunonegative/CRH neurons were identified when a brown chromogen product could be identified in the cytoplasm with an empty nucleus in the same cell. The observer was blind to the treatment groups during image analysis. Numbers of CRH- and ERbeta immunopositive or immunonegative neurons were averaged from sections from each rat that were selected from 3 comparable areas of the PVN corresponding to the regions between Bregma -1.4 to Bregma -1.8 (all anatomical references are based on the Paxinos and Watson Atlas (Paxinos 1998)). The statistical analysis of these means is described in detail in Chapter 5.
CHAPTER THREE
EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS RESPONSE TO CORTICOTROPIN RELEASING HORMONE STIMULATION

3.1 Introduction

This chapter is excerpted from a published work by Sithisarn et al (Sithisarn, Bada et al. 2008). As mentioned in the introduction, the prevalence of non-medical use of pain relievers during pregnancy continues to increase and is a major public health problem, with oxycodone being one of the most widely abused pain relievers. Oxycodone is a strong, semi-synthetic, mu-and kappa opioid agonist with an oral potency that is approximately twice that of oral morphine (Benziger, Miotto et al. 1997). It is estimated that 1.2 % of pregnant women abuse pain relievers annually, a higher proportion than the 0.3 % of women who use cocaine during pregnancy (SAMHSA 2006). Earlier human studies investigated the effects of prenatal exposure to heroin and methadone on neurobehavioral outcome. Although the effects of prenatal opiate exposure on neurobehavior were subtle, e.g. crying with more short utterances and more hyperphonation, these effects could be observed at one month of age in a poly-drug model and reflected neurobehavioral vulnerability (Lester, Tronick et al. 2002). The reported effects of prenatal opiate exposure on motor, mental, and behavioral development in older children have been conflicting, with more pronounced abnormal findings in the studies done in prenatal methadone exposed children, and more subtle or no difference found in the studies conducted in the poly-drug use models (Strauss, Andresko et al. 1976, Strauss, Lessen-Firestone et al. 1979, Johnson, Diano et al. 1984, Hans 1989, van Baar, Fleury et al. 1989, Messinger, Bauer et al. 2004, Bada, Das et al. 2007).
It is well-established that child behavior problems are associated with changes in circulating cortisol (van Goozen, Matthys et al. 1998, van Goozen, Matthys et al. 2000, Bauer, Quas et al. 2002). For example, lower baseline cortisol levels have been reported in children with behavior problems, and the increase in cortisol after stress appeared to be exaggerated. Internalizing behavior has been associated with high adrenocortical and sympathetic activation in children, but low activation is associated with externalizing and aggressive behavior (Bauer, Quas et al. 2002). Based on these findings, the dysfunction of the hypothalamic-pituitary axis (HPA) could be, at least in part, an underlying cause of behavioral problems during childhood, including those found in children who have been exposed to prenatal opiates.

In animal studies, prenatal morphine treatment causes neurobehavioral changes and alterations in function of both the hypothalamic-pituitary-adrenal and gonadal axes (Lesage, Bernet et al. 1996, Lesage, Grino et al. 1998, Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). However, limited data are available on the effects of prenatal oxycodone on the offspring’s stress response. Since prenatal use of opiates or other drugs in pregnancy can influence in utero brain development, including the central nervous control of the stress axis, we explored the effect of prenatal oxycodone exposure on the HPA function using an animal model. We also explored whether these alterations in the HPA function might be sex-specific. No data are available regarding the effect of perinatal opiate exposure on the stress response during pharmacological stress with corticotropin realeasing hormone (CRH) injection. However, previous studies in prenatal morphine exposure models indicate that prenatal morphine exposure is associated with decreased ACTH response during restraint stress tests conducted on male and female offspring (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). We hypothesize that perinatal oxycodone
exposure will have an effect on the HPA development, which will be manifested as a blunted ACTH and/or corticosterone response to stress during late adolescence after CRH injection.

3.2 Methods

3.2.1 Animals and prenatal treatments

Fourteen timed pregnant Dark Agouti (DA) rats (Harlan, Indianapolis, IN) weighing 170-189 g were housed individually in maternity cages on gestational day (GD) 3. All animals in this study were treated as described in detail in Chapter 2, i.e., they received a right atrial cannula on GD 5-6 through which they received daily injections from GD 8-21 and on PD 1, 3 and 5. The pregnant rats were randomly assigned to either a control (CON, saline, 1 ml/kg, n=8) or oxycodone (0.8 mg/kg, n=6) treatment group.

DA rats were chosen in preference to Sprague-Dawley (SD) rat in this experiment because the DA rat is genetically deficient in the CYP2D1 enzyme that catalyzes the O-dimethylation of oxycodone to oxymorphone. This dimethylation with subsequent glucuronidation to oxymorphone-3-glucuronide accounts for less than 5% of an oxycodone dose in humans, and therefore, at the time, it was assumed that the DA rat may be a closer model to the human than the SD rat (Cleary, Mikus et al. 1994). The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

3.2.2 Corticotropin Releasing Hormone (CRH) versus NSS stimulation tests

On PD 43, male and female pups were randomly selected from each oxycodone- or saline-exposed litter and assigned to either a CRH or NSS group for subsequent stimulation tests. To eliminate litter effects, no 2 animals in a given group were
littermates (Chapman and Stern 1979). Each rat was fitted with a right atrial cannula as described in Chapter 2. The cannulae were flushed daily for the next 2 days. The rats were removed from their cage and held gently while flushing the cannula or obtaining blood samples. The rats were transported to the lab on the day before the experiment for acclimatization. On PD 45 between 1000 and 1500 hours, either ovine CRH (National Hormone and Peptide Program, CA) (10 µg/kg in NSS, 0.3 ml) or 0.3 ml NSS was injected as an IV bolus (over ~30 sec) through the cannula. Blood samples (0.3 ml) for determination of plasma ACTH and CORT were obtained via the cannula immediately before (0 min baseline) and at 15, 30 and 60 minutes after CRH injection. To prevent stress from loss of blood volume, 0.3 ml NSS was administered after each sample. Blood samples were collected into tubes containing 1.2 mg EDTA, and immediately refrigerated (10 ° C). Once all samples were obtained, the plasma samples were separated by centrifugation and stored at -70 ° C for future assays (Kamilaris, DeBold et al. 1991). The prenatal treatment model and CRH/ NSS tests are outlined in Figure 3.1.

3.2.3 Assay of ACTH and CORT Hormones.

Both ACTH and CORT levels were determined by radioimmunoassay (RIA) (MP Biomedicalals, Orangeburg, NY). The samples were diluted to fit within the linear portion of the standard curves. The sensitivities, based on the lowest dose of standard, were 14 pg/ml and 25 ng/ml for the ACTH (n=2) and CORT (n=2) assays, with inter-assay coefficients of variation of 5.0 ± 1.1 % for the CORT assays and 6.6 ± 6.6 % for the ACTH assays.
3.2.4 Data analysis and statistical methods

Data were analyzed using SAS 8.2 (SAS Institute, Cary, NC). Differences were considered significant if p<0.05. Two-sample t-tests, Chi square tests, or Fisher’s exact tests were performed for comparison of baseline characteristics. To take intra-subject correlation into account, we used a linear mixed model to study the four-way (perinatal exposure (oxycodone vs. CON), CRH vs. NSS stimulation test, time from injection, and sex) main effects on plasma ACTH and CORT concentrations. Post hoc comparisons were performed in the linear mixed model on significant main effects to investigate the magnitude and duration of alterations in ACTH and CORT.
3.3 Results

3.3.1 Parturition, litter size and body weights

There were no differences between the oxycodone-exposed and CON pups in timing of parturition, litter size and body weight, either male or female, from birth to PD 32 (p>0.05). For the male pups in oxycodone (n=9) and CON groups (n = 13), mean birth weights ± SEM were 5.41 ± 0.31 g and 4.92 ± 0.11 g respectively. For the female pups, mean birth weights ± SEM were 4.98 ± 0.15 g and 4.59 ± 0.17 g respectively for the oxycodone (n=11) and CON (n=10) groups. Neither were there differences in body weights of the pregnant rats between the two groups before or after delivery.

During adolescence on PD 45, in the oxycodone group (n=20), 11 rats received CRH (5 males, 6 females) and 9 rats received NSS (4 males, 5 females). In the CON group (n=23), 14 rats were treated with CRH (8 males, 6 females) and 9 with NSS (5 males, 4 females). Mean (± SEM) plasma levels of ACTH and CORT concentrations are shown in Figures 3.2 and 3.3.

3.3.2 Plasma ACTH levels

There was no effect of either prenatal exposure to oxycodone or sex on mean basal plasma ACTH concentrations (Figure 3.2). NSS had no effect on plasma ACTH levels; however CRH elicited an increase in all four groups (p<0.001) (Figure 3.2). In males, prenatal oxycodone exposure was associated with a greatly enhanced response to CRH such that the peak was more than 2-fold greater than that in CON males (p<0.001, Table 3.1), and occurred about 15 minutes later (p<0.001, Table 3.1 and Figure 3.2). This response was so exaggerated that plasma ACTH levels were still above baseline 60 minutes after CRH administration, when levels in the CON males were not different from the baseline (p<0.001, Table 3.1). In contrast, oxycodone had no
effect on the ACTH response to CRH in females, in which the peak level occurred at 15 minutes after CRH injection and was similar to that in the CON females. However in females, the increase in plasma ACTH levels in response to CRH was prolonged or continued to be significantly higher than baseline in both oxycodone and control groups at 60 min (60 min vs. baseline, p=0.04 in oxycodone, p<0.001 in CON, Table 3.1).

3.3.3 Plasma CORT levels

Neither prenatal oxycodone exposure nor sex had an effect on basal CORT concentrations (Figure 3.3). Although CORT levels increased in response to injection of both NSS and CRH, CRH stimulation induced a greater elevation in CORT as compared with NSS stimulation collapsed across treatment and gender (p< 0.001). In contrast to the effect of prenatal oxycodone exposure on the ACTH response to CRH in males, there was no effect of prenatal oxycodone exposure on the CORT responses to either NSS or CRH. As expected, females had greater CORT responses to both NSS and CRH than males (female-male difference: at 15 min=193.08±75.94 ng/ml, p=0.01; at 30 min=318.77±75.94 ng/ml, p<0.001; at 60 min= 272.68±75.94, p<0.001).

3.4 Discussion

These results demonstrate that perinatal oxycodone exposure increases the pituitary, but not the adrenal postnatal response to CRH in late adolescent male rats, but not in females. Thus, although CORT levels were not different between the oxycodone and CON groups, the amplitude of the ACTH response to CRH was greatly enhanced in males but not in females. Moreover, the pattern of response to CRH was altered by prenatal oxycodone exposure; the peak ACTH response occurred later and the response was prolonged. The CORT response to stress was also sex-specific; females
had higher CORT levels than males. Our findings are consistent with previous findings that CRH stimulation leads to a greater response of HPA-axis than injection of NSS (Kamilaris, DeBold et al. 1991, Walker and Dallman 1993) and that CORT levels in female rats are usually greater than in the males, as demonstrated in several different models (McCormick, Smythe et al. 1995, Nock, Cicero et al. 1998, Koehl, Darnaudery et al. 1999).

The stimulatory effect of perinatal exposure to oxycodone on CRH-induced ACTH release is in contrast to previous observations of a suppressive effect of prenatal morphine exposure on ACTH in male and female offspring during restraint stress tests (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). In addition to sex, differences in mechanisms of action of drugs, dosage, timing of gestation and length of exposure, and many other factors can influence fetal HPA-axis development and function (Vathy 2002). Both morphine and oxycodone are opioid receptor agonists, but the intrinsic antinociceptive effects of morphine are mediated primarily by mu-opioid receptor (MOR). However, the effects of oxycodone, having a relatively low affinity to MOR (Horan, de Costa et al. 1991), are also mediated through ketocyclazone or kappa-opioid receptor (KOR); these effects are markedly attenuated by norbinaltorphimine, a KOR selective antagonist (Ross and Smith 1997). Moreover, the characteristics of the degree of antinociception versus time profiles for oxycodone are similar to those observed following intracerebroventricular administration of known KOR agonists (Leighton, Rodriguez et al. 1988, Horan, de Costa et al. 1991, Ross and Smith 1997) but are completely different from those observed following morphine administered by a similar route (Leow and Smith 1994, Ross and Smith 1997). The opiate tolerance, abstinence-induced hypersecretion of CORT and alterations in the responsiveness of the HPA-axis after chronic exposure to MOR or KOR agonists are also opioid receptor
specific (Ignar and Kuhn 1990). Thus, the difference between the effects of prenatal oxycodone exposure and those resulting from prenatal morphine exposure may be due to mediation by different subtypes of opioid receptors.

A potential role for KOR in activation of the fetal HPA-axis has been studied in the ovine fetus (Taylor, Wu et al. 1996, Szeto 2003). When U50,488H, a selective KOR agonist, was administered directly to the ovine fetus, it produced a significant increase in plasma ACTH and CORT that was completely blocked by naloxone, and partially blocked by antagonists of CRH and arginine vasopressin (Szeto 2003). Interestingly, the combined blockade of CRH and arginine vasopressin receptors failed to completely block the action of this selective KOR agonist in the fetus, suggesting the possible involvement of other hypothalamic secretagogues such as enkephalin, galanin and dynorphin (Szeto 2003, Watts 2005, Elson and Simerly 2015). To date, the long term effects of KOR agonists on the developing HP-axis have not been well established.

The timing of prenatal exposure might be another reason for differences between our findings and previous studies on prenatal morphine exposure. Our rats were exposed to oxycodone at an earlier time with a longer period of exposure until the postnatal period, while in other studies pregnant rats were exposed for one week from GD 11 to 18 (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). At the earlier period in gestation, fetal cortico-stimulating activity is mostly independent of the fetal hypothalamus, while between days 19 and 21, the hypophyseal corticotrophic activity is mostly under the hypothalamic control (Dutriez-Casteloot, Bernet et al. 1999). In addition, if the opiate administration is terminated abruptly after delivery, suppression or alteration of the HPA-axis response may be an effect of drug withdrawal (Zagon and McLaughlin 1992). Withdrawal manifestations have been observed in pups with cessation of opiate treatment (Hamilton, Harris et al. 2005) even when fostered from
birth. This possibility was lessened in our study by administering oxycodone on PD 1, 3 and 5, as a way of weaning the pups off daily dosing. In spite of its short half-life (Bostrom, Simonsson et al. 2005), oxycodone has been shown to be highly distributed in the brain by its high influx and binding to brain tissue (Bostrom, Simonsson et al. 2006), and every-other-day administration may have been sufficient to have minimal exposure to oxycodone, minimizing withdrawal manifestations. Moreover, we found no differences in weights of both the dams and the pups between oxycodone and CON groups in the postnatal days through weaning, which suggested that nutritional status of the pups was comparable even with no fostering. Weight loss in the pups has been noted as a sensitive indicator of withdrawal (Lichtblau and Sparber 1982, Lichtblau and Sparber 1983).

That we did not foster the pups after birth is a limitation of our study. It has been described that the poor quality of parental rodent care in the postnatal period has a long-term effect on HPA-axis, neurobiological, and psychosocial development (Huot, Gonzalez et al. 2004, Michel and Tyler 2007). However, our study was an initial attempt to examine the effect of perinatal oxycodone administration on the HPA-axis to include the early postnatal days in the rats (Dent, Smith et al. 2000, Matthews 2002). We fostered the litters in our subsequent experiments as will be described in the next Chapter.

Interestingly, although oxycodone increased plasma ACTH in male rats after CRH stimulation, the levels of CORT were not greater in oxycodone compared to CON rats as could be expected. This finding needs to be examined in the context of a possible decrease in CORT negative feedback as a result of prenatal opiate exposure. The role of the negative feedback mechanism has been examined previously using the dexamethasone (DEX) suppression test. In adult male rats, DEX pretreatment could
suppress the restraint stress-induced increase in plasma ACTH and CORT concentrations in a dose-dependent manner, regardless of prenatal morphine exposure (Rimanoczy, Slamberova et al. 2003). In contrast, a higher dose of DEX was required for suppression in morphine-exposed adult female rats (Slamberova, Rimanoczy et al. 2004, Slamberova, Rimanoczy et al. 2005). Such impairment of the negative feedback action of CORT may have caused the elevated and prolonged ACTH levels in the oxycodone-exposed males in our study. In a prenatal stress model in which prolonged restraint was used to stress the mothers, the release of CORT in male and female offspring in response to postnatal environmental stress was greater and of a longer duration than that in controls (Vallee, MacCari et al. 1999, Richardson, Zorrilla et al. 2006, Weinstock 2007). This was associated with a decrease in the number of hippocampal glucocorticoid (GR) and mineralocorticoid receptors (MR) indicating, as well, impairment in the feedback regulation of the HPA-axis (Weinstock, Matlina et al. 1992, Maccari, Darnaudery et al. 2003, Weinstock 2007). The effects of prenatal opiate exposure on the feedback mechanisms have been touched on previously in a prenatal morphine exposure model. Female rats prenatally exposed to morphine had a decreased ACTH response to restraint stress test (RST) with altered sensitivity of negative feedback mechanism as tested by dexamethasone suppression (Slamberova, Rimanoczy et al. 2004). However, these changes in the sensitivity of the negative feedback appeared unaltered in the male rats exposed to morphine prenatally, as the DEX suppression was not changed (Rimanoczy, Slamberova et al. 2003). Whether these findings in prenatal morphine models involved alteration in MR or GR expressions was not studied. Therefore, we will further explore the effects of perinatal oxycodone exposure on the HPA-axis using RST as a stress paradigm and will explore possible changes in the feedback circuitry that may explain changes in the HPA-axis functions, as will be described in Chapters 4 and 5.
Additionally, the increased plasma ACTH in oxycodone-exposed male rats after CRH stimulation with minimal increase in CORT levels raises the possibility of adrenal desensitization to ACTH. Previous studies in rats have shown that there are long term effects of stressors, such as restraint, on HPA function (Marti, Garcia et al. 2001, Armario, Marti et al. 2004). Exposure to restraint 9-10 days after an initial or first exposure resulted in a decrease in CORT response compared to that elicited by the first exposure to this stressor. However, ACTH responses to restraint were not different between the first and subsequent exposures. Thus, lower ACTH levels did not cause the lower CORT response on subsequent stress exposure (Marti, Garcia et al. 2001, Armario, Marti et al. 2004). In another study using immobilization stress, blockade of glucocorticoid receptors with the antagonist mifepristone before the first exposure to immobilization did not modify the effects on the ACTH response during a second immobilization exposure, but did partially block the effects on the CORT response (Armario, Marti et al. 2004). In addition, rats given MK-801, an NMDA (N-Methyl-D-aspartate) receptor antagonist, before the first immobilization stressor, had a less marked decline in CORT response with no modification of the decline of plasma ACTH (Armario, Marti et al. 2004) during the second immobilization experience. These findings support the hypothesis that previous experience with stress can inhibit adrenocortical responsiveness to circulating ACTH, suggestive of adrenal desensitization (Armario, Marti et al. 2004). The mechanisms underlying the dissociation of adrenal responses from ACTH levels are unclear. Possible alternative explanations include, for example, the roles of NMDA receptors or circulating signals from sympathetic nerves directly affecting the adrenal gland (Vinson, Hinson et al. 1994, Armario, Marti et al. 2004). Another possibility that could explain the absence of an enhanced CORT response to elevated ACTH is that the relatively high dose of CRH in our experiment achieved the maximal response from the adrenal gland.
Prenatal morphine exposure alters the ACTH and CORT responses to stress and the sensitivity to negative feedback of glucocorticoids differentially according to sex. Prenatal stress also has sex-specific effects on postnatal HPA-axis function (McCormick, Smythe et al. 1995, Koehl, Darnaudery et al. 1999, Weinstock 2007). Similarly, the foregoing findings indicate that prenatal oxycodone also has sex-specific effects on the ACTH and CORT responses to CRH stimulation. The elevated CORT levels that we observed in females compared to those in males are most likely due to the sex differences in circulating levels of estradiol and testosterone, which have myriad effects on the HPA-axis during fetal and postnatal life. It appears that testosterone can act to inhibit HPA function, whereas estrogen can enhance HPA function (Handa, Burgess et al. 1994, Viau 2002, Seale, Wood et al. 2004, Seale, Wood et al. 2004, Lund, Hinds et al. 2006).

In summary, we have described that the HPA-axis responses to CRH administration in prenatally oxycodone-exposed experimental animals are altered. Prenatal oxycodone increases the ACTH, but not the CORT response to CRH stimulation in late adolescent male, but not female offspring. Unlike morphine acting mainly on MOR, the effects of oxycodone are also mediated through KOR, and further studies are needed to clarify the mechanisms underlying these effects of perinatal oxycodone exposure. These results reflect responses following stress as an acute CRH challenge; however, HPA function in prenatally exposed animals may be different in the presence of different types of stress, especially psychological stress as seen more commonly in children exposed to maternal substance abuse in utero. Therefore we will study the effect of perinatal oxycodone exposure on the HPA-axis response to stress using a different stress paradigm, a restraint stress test that will involve the limbic control of the HPA-axis, as will be described in Chapter 4. We will further study the mechanistic
changes in the circuitry controlling the HPA-axis that may explain the enhanced ACTH response to stress as will be described in Chapter 5.
Figure 3.1: Perinatal oxycodone treatment model and postnatal CRH and NSS stimulation test
Figure 3.2: Effect of prenatal exposure to oxycodone (OXY) on mean (± SEM) plasma ACTH in response to postnatal CRH or NSS administration. The male (left panel) and female (right panel) offspring were exposed in utero to either OXY (in blue) or NSS vehicle (CON, in black), and on postnatal day 45, were treated IV with either CRH (solid lines) or NSS (dashed lines) just after the first sample at $t = 0$. 
Figure 3.3: Effect of prenatal exposure to oxycodone (OXY) on mean (± SEM) plasma corticosterone concentrations, in response to postnatal CRH or NSS administration. The male (left panel) and female (right panel) offspring were exposed in utero to either OXY (blue lines) or vehicle (CON, black lines), and on postnatal day 45, were treated IV with either CRH (solid lines) or NSS (dashed lines) just after the first sample at t=0.
Table 3.1: Post hoc comparisons of plasma ACTH means by exposure (oxycodone vs. control group), test (CRH vs. NSS), and sex

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>Test</th>
<th>N</th>
<th>Peak time (min)</th>
<th>Plasma ACTH (ng/ml); mean ± SEM</th>
<th>Changes peak vs. baseline levels</th>
<th>Changes 60 min vs. baseline levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>Oxycodone</td>
<td>CRH</td>
<td>5</td>
<td>30</td>
<td>8241.3 ± 1159.6 **</td>
<td>3720.2 ± 1004.5 **</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>CRH</td>
<td>8</td>
<td>15</td>
<td>2949.9 ± 845.2 **</td>
<td>868.3 ± 705.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference between OXY and control groups after CRH</td>
<td></td>
<td></td>
<td></td>
<td>4977.2 ± 1238.1 **</td>
<td>2437 ± 990.0 *</td>
<td></td>
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<tr>
<td></td>
<td>Oxycodone</td>
<td>NSS</td>
<td>4</td>
<td>30</td>
<td>426.3 ± 1043.7</td>
<td>332.7 ± 960.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>NSS</td>
<td>5</td>
<td>15</td>
<td>842.8 ± 858.8</td>
<td>240.3 ± 858.8</td>
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<tr>
<td></td>
<td>Difference between OXY and control groups after NSS</td>
<td></td>
<td></td>
<td></td>
<td>388.4 ± 1047.9</td>
<td>120.6 ± 964.8</td>
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<tr>
<td></td>
<td>Female</td>
<td>Oxycodone</td>
<td>CRH</td>
<td>6</td>
<td>15</td>
<td>2192.5 ± 976.5 *</td>
<td>1819.9 ± 885.9 *</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>CRH</td>
<td>6</td>
<td>15</td>
<td>3089.3 ± 885.4 **</td>
<td>2937.7 ± 826.3 **</td>
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<tr>
<td></td>
<td>Difference between OXY and control groups after CRH</td>
<td></td>
<td></td>
<td></td>
<td>772.8 ± 1094.5</td>
<td>993.8 ± 963.3</td>
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<tr>
<td></td>
<td>Oxycodone</td>
<td>NSS</td>
<td>5</td>
<td>30</td>
<td>686.5 ± 858.8</td>
<td>175.6 ± 858.8</td>
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<tr>
<td></td>
<td>Control</td>
<td>NSS</td>
<td>4</td>
<td>30</td>
<td>991.3 ± 960.2</td>
<td>612.2 ± 960.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference between OXY and control groups after NSS</td>
<td></td>
<td></td>
<td></td>
<td>290.8 ± 964.8</td>
<td>422.8 ± 964.8</td>
<td></td>
</tr>
</tbody>
</table>

Shown are the time of peak (15, 30, or 60 min) levels of plasma ACTH (pg/ml) after CRH or NSS test expressed as change in levels (peak minus baseline and levels at 60 min minus baseline), and differences of these changes in measurements between oxycodone and control groups for each sex.

* P-value < 0.05
** P-value < 0.001
CHAPTER FOUR
EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS RESPONSE TO RESTRAINT STRESS TEST

4.1 Introduction

As we have earlier demonstrated (Chapter 3 and (Sithisarn, Bada et al. 2008)), prenatal oxycodone exposure (POE) is associated with an enhanced peak ACTH response to CRH stimulation in the male offspring but no effect on the ACTH response in females. The CORT response to CRH was not different between control and POE rats. These results demonstrate that POE enhances pituitary response. We utilized the CRH stimulation test in our previous experiment to assess the HPA axis response; however, as it is a direct pharmacologic stimulation to the pituitary gland, it may not constitute a physiologic challenge to the whole complex HPA-axis and limbic system. Therefore in this chapter, we have tested another stress paradigm to include psychological stress. Existing experimental approaches to stress induction, besides pharmacologic models, include models of social conflict and disruption, genetic models and naturalistic models of survival threat, with deprivation paradigms being one of the most widely used (Patchev and Patchev 2006). Deprivation or restriction of freedom and exploration, known as restraint stress, was selected as the experimental stressor in this study. The restraint stress test (RST) has been one of the most commonly employed techniques to induce classical stress-related behavioral, biochemical and physiological responses in laboratory animals including rats (Jaggi, Bhatia et al. 2011, Campos, Fogaca et al. 2013). Restraint stress produces inescapable physical and psychological stress and it consistently induces ACTH and CORT responses as shown in many studies (McCormick, Smythe et al. 1995, Gadek-Michalska, Spyrka et al. 2013, Drouet,
Fauvelle et al. 2015). Restraint stress is a modified form of immobilization in which the animals are not adhesively taped to a board but are not allowed to move for a specified period of time (Bali and Jaggi 2015). By placing the test animal in a well-ventilated plastic tube or a wire-mesh container, the animal's range of movement is severely limited (Southwick, Bremner et al. 1994). The duration of a RST may vary to investigate different aspects of stress (Bali and Jaggi 2015). In this study we decided to acutely restrain the rat in a restrainer for one hour and measure ACTH and CORT concentrations during and after the restraint for up to 1.5-2 hrs after the beginning of the restraint. We hypothesized that POE offspring will have increased ACTH and/or CORT responses to stress during RST in comparison to the control group. We also hypothesize that there may be gender differences in the dysregulation of the HPA axis as had been observed earlier. Previous studies demonstrated that prenatal morphine exposure suppresses the ACTH response, but has no effect on the CORT response during RST in both male and female rats (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). However, there has been no previous study to examine the effects of prenatal exposure to oxycodone on the response to RST. This experiment will fill this gap in our knowledge.

Our previous experiments had certain limitations which we modified in this study. First, previously we had performed cannulation surgery on rat dams on GD 5-6. Although at this early period in gestation, the effect of surgical stress on the fetal adrenal cortex is mostly independent of the fetal hypothalamus (Dutriez-Casteloot, Bernet et al. 1999), the stress from cannulation surgery this early in gestation could potentially impact maternal stress levels and thereby potentially affect the development of the HPA axis. Hence in the current experiment we performed the cannulation surgery in virgin female rats before mating and conception. Second, in the previous study, we did not foster the
pups after birth. Rat dams receiving oxycodone treatment may have poor nurturing behavior. This possibility is based on the finding that exposure of female rats to an opiate like morphine during their adolescent period can induce changes in subsequent maternal care including decreased nursing frequency and pup grooming and can alter the behavioral phenotype of subsequent offspring (Johnson, Carini et al. 2011). It has been well-described that maternal nurturing behavior can greatly impact stress responsiveness and neuro-behavior in the offspring (Champagne, Francis et al. 2003, Fish, Shahrokh et al. 2004, Champagne, Bagot et al. 2008). Thus in this experiment we have used foster rat mothers to avoid these problems. Third, we did not test the dose effects of oxycodone in the previous study; therefore in this protocol we administered two different doses of oxycodone as described below.

4.2 Material and Methods

4.2.1 Experimental Design: Animals and prenatal treatments.

The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee. Virgin SD rats (Harlan, Indianapolis, IN) were used in this Chapter as well as Chapter 5, 6 and 7 instead of DA rats, as described in Chapter 2. Rats weighing 194-223 g were housed individually at 22-25° C and maintained in a 14L:10D photoperiod (lights on at 0500 h) with regulated 30-70% humidity. Rat chow and water were provided ad libitum.

The animal strain and prenatal treatments described in this chapter were also used in Chapter 5, 6 and 7. Once released from quarantine, the females were fitted with a right atrial cannula. This cannula is connected to a subcutaneous access port implanted between the shoulder blades. The rats were allowed to recover for 1 week (see more details in Chapter Two). The cannulae were flushed daily via the
subcutaneous port with sterile heparinized saline (0.4 cc, 100 IU/ml) until GD 8. To determine estrous cycles vaginal lavages were obtained daily (see details in Chapter Two). Each female was group housed with a proven breeder male for breeding one week after cannulation. GD 0 was designated as the day that sperm were detected in the vaginal smear, and the females were individually housed thereafter. Foster females were bred at the same time and remained untreated throughout their gestation.

The experimental dams were divided into 3 treatment groups that received either oxycodone (Mallinckrodt, St. Louis, MO) at a low (OXY-L, 0.5 mg/kg/day, number of dams = 5) or high dose (OXY-H, 2.0 mg/kg/day, number of dams = 12) or an equivalent volume of vehicle (CON, normal saline solution (NSS, 1.0 ml/kg/day), number of dams = 12) from GD 8-21. These solutions were slowly injected i.v. over 10 minutes via the s.c. access port. The lower dose was slightly lower than that used in our previous protocol (Chapter 3) (0.8 mg/kg). The higher dose was selected to be well tolerated by the dams without loss of pregnancy based on our preliminary results.

On PD 1 the pups were counted and weighed. Oxycodone or NSS was also administered on PD 1, 3, and 5 at the same dosages delivered during gestation because brain development during PD 1, 3 and 5 in neonatal rats is similar to that during the third trimester of human gestation, and to prevent maternal withdrawal symptoms that may affect maternal nursing behavior. On PD 2 all litters were adjusted to contain 10-11 pups with equal numbers of male and female pups when possible. At 5 pm on PD 5, all pups in each litter were transferred to a foster dam. The pups were weighed daily and weaned at PD 25, when they were separated by sex.
4.2.2 Restraint Stress Test (RST)

Restraint stress in this study was achieved by confining the rats in flat bottomed cylindrical acrylic restrainers (size measuring 50.8 x 108 x 171.5 mm for rats weighing 250-500 g; 44.5 x 76.2 x 127 mm for rats weighing 125-250 g) (Harvard Apparatus, Holliston, MA) for 1 h between 8 am and 1 pm. Each rat was gently removed from a home cage and placed in a restrainer with lengths of rubber tubing placed to the sides of rat’s body to prevent turning around. After the 60-minute blood sample was obtained, each animal was released back to its own cage. The rats were gently held on the experimenter’s lap for the subsequent blood draws.

The RSTs were performed on PD 50-60. CON (n=47, 23 males, 24 females), OXY-L (n=28, 14 males, 14 females) and OXY-H offspring (n=44, 20 males, 24 females), were randomly selected to endure acute restraint stress for one hour. Blood samples were collected via tail nick before and 15, 30, and 60, 90, 120, 180 (and 240 in females) minutes after confinement. At each time point, blood samples (120 µl X 2 tubes, for ACTH and CORT respectively) were collected into microtubes containing 1.2 mg EDTA, and immediately refrigerated (10º C). Once all samples were obtained, the plasma samples were separated by centrifugation, and stored at - 80˚ C for future assay of plasma ACTH and CORT concentrations by RIA. The perinatal oxycodone treatment model and postnatal restraint stress test are outlined in Figure 4.1.

4.2.3 ACTH and Corticosterone Assay.

Both ACTH and CORT levels were determined by radioimmunoassay (RIA) (MP Biomedicals, Orangeburg, NY) (details in Chapter Two). We performed 7 ACTH assays and 7 CORT assays. The sensitivities, based on the lowest dose of standard, were 14 pg/ml and 25 ng/ml for the ACTH (n=7) and CORT (n=7) assays respectively with inter-
 assay coefficients of variation of 3.4 ± 2.8 % for the CORT assays and 4.8 ± 1.5 % for the ACTH assays.

4.2.4 Statistical Analyses

A multivariate mixed linear regression model, using a random litter effect and an unstructured covariance across time, was fitted using restricted maximum likelihood to test for the impact of group, time, and any potential interactions these variables have on the mean values for the natural log of ACTH and CORT levels. The natural log was used to reduce the influence of outliers and skewed data. The Kenward and Roger (1997) degrees of freedom were used. Variables were treated as categorical, and backward elimination at the 5% significance level was utilized to eliminate interactions (Kenward and Roger 1997). When undetectable, values under 25 ng/ml were given values of 20 ng/ml. Analyses based on multiple imputations for these values were also conducted and gave similar results; therefore, the undetectable values were not included in the analysis. Tests were two-sided and were conducted in SAS version 9.4 (SAS Institute, Cary, N.C.). Male and female results were analyzed separately as it is well known that females have higher ACTH and CORT responses to stress compared to the males (McCormick, Smythe et al. 1995, Nock, Cicero et al. 1998, Koehl, Darnaudery et al. 1999).
4.3 Results

4.3.1 Plasma ACTH

In males, restraint stress significantly increased ACTH levels from baseline, which were highest at 30 min in all treatment groups. There were no differences in plasma ACTH levels among treatment groups (Figure 4.2, left panel).

Females also responded to RST with increased ACTH levels, the maximum response occurring at 15 minutes in all treatment groups. As in the males, plasma ACTH levels did not differ among the three treatment groups (Figure 4.1, right panel). As has been previously reported, female rats had higher ACTH responses to restraint stress than males (time X gender, p = 0.01) (Figure 4.2, left and right panels).

4.3.2 Plasma Corticosterone

RST increased circulating CORT concentrations in all treatment groups (Figure 4.3, left and right panels). CORT concentrations were not different among groups in the males (Figure 4.3, left panel).

In females, the CORT response was statistically higher in OXY-L females than in OXY-H females over time which was apparent at 30 and 60 minutes (p= 0.032 and p=0.036 respectively). Although CORT levels in female OXY-L were not statistically higher than female CON when multivariate mixed linear regression model was applied, the patterns of CORT concentrations in response to RST in female CON and OXY-H were quite similar; thus CORT levels in OXY-L appeared higher than CON as well, as shown in Figure 4.3, right panel. In agreement with previous reports, baseline CORT levels and responses to restraint were higher in females than in males (p = 0.027; Figure 4.3, left and right panel).
4.4 Discussion

In agreement with the findings from our previous experiment, perinatal oxycodone exposure is associated with an enhanced HPA-axis response to stress (Sithisarn, Bada et al. 2008). However, in that earlier study, we found that the ACTH response to CRH stimulation in oxycodone-exposed males was enhanced without significant differences in CORT response, while the current experiment demonstrated that the CORT, but not ACTH, response to RST was increased in the females exposed to oxycodone in utero. POE did not change the baseline ACTH and CORT levels, similar to the results of prenatal morphine exposure model from Slamberova et al and Rimanoczy et al (Slamberova, Rimanoczy et al. 2004).

Previous studies by others have reported mixed effects (either suppressive or no effects) of perinatal opiate exposure on the HPA-axis function of adult animals, taking into consideration (see below) that the models were not exactly the same. Rimanoczy et al and Slamberova et al reported a suppressive effect of prenatal morphine exposure on the HPA-axis response to RST (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). In their model, pregnant female Sprague-Dawley rats were treated with subcutaneous morphine injections twice daily from GD 11-18. The first three doses were 5 mg/kg and all subsequent doses were 10 mg/kg (Rimanoczy, Slamberova et al. 2003). The results showed that in the males, prenatal morphine exposure suppressed the RST-induced ACTH response when compared to saline-injected animals or controls that did not receive any prenatal injections. The patterns of the ACTH response were also different among groups; the ACTH response in morphine-exposed males remained elevated after 80 minutes post RST without a declining pattern, as compared to the saline or control groups. In contrast to ACTH, CORT responses to RST
were not different between the groups (Rimanoczy, Slamberova et al. 2003). In female offspring, prenatal morphine exposure also attenuated the ACTH increase in response to RST during both the diestrous and proestrous stages of the estrous cycle. Similar to the results in the males, there was also no effect of prenatal morphine on the CORT response to RST (Slamberova, Rimanoczy et al. 2004). The baseline ACTH and CORT concentrations were not different among treatment groups in both males and females. A study by Dutriez-Casteloot et al, on the other hand, did not find significant effects of prenatal morphine exposure on the HPA-axis. In their model, pregnant female Wistar rats received subcutaneous injections of morphine (10 mg/kg) twice a day from GD 11-18. Under resting conditions, prenatal morphine exposure did not change the activity of the HPA-axis, including hypothalamic CRF content, plasma ACTH concentrations, adrenal weights and adrenal CORT content, and plasma CORT levels in adult (80-90 day-old) male offspring (Dutriez-Casteloot, Bernet et al. 1999).

Differences in the results from our study and others could be due to many factors, including the specific opiates tested, the dose, the route and timing of administration during brain development, and the species used in the model, among others. Thus these methodology issues make it difficult to compare the results from different studies. The most likely explanation for the differences in the results is that prenatal exposure to different subtypes and classes of opiates used in the models could cause different effects on the HPA-axis. Morphine is mainly a µ-opioid receptor (MOR) agonist, but also binds with lower affinity to kappa receptor (κ-receptors, KOR) (Trescot, Datta et al. 2008). Oxycodone has activity at multiple receptors but mainly at the KOR, and to a lesser degree at MOR- and Delta- opioid receptors. The major metabolite of oxycodone, oxymorphone, has high affinity to MOR. Although acute administration of either MOR agonists (such as morphine and fentanyl) or κ-receptor agonists (such as
U50,488) can stimulate the HPA-axis leading to an increase in plasma CORT levels in a receptor-dependent manner, each receptor agonist has different effects when administered chronically (Pechnick 1993). Previous data suggest that the HPA-axis develops tolerance, but not withdrawal after chronic kappa agonist exposure; in contrast, it develops elevated CORT secretion after withdrawal from chronic exposure to MOR agonist (Ignar and Kuhn 1990). Thus the effects of prenatal exposure to both oxycodone, which has high activity on kappa opioid receptors, and its metabolite oxymorphone, which acts on mu receptors in the HPA-axis, could be complicated.

In addition, the localization of each opioid receptor in brain regions is quite distinctive. While distributed in many areas of the brain, the majority of MOR receptors were observed in somatodendritic profiles in the rat periaqueductal gray and hippocampus (Vuong, Van Uum et al. 2010, Bodnar 2012). Medial prefrontal cortical innervation of the intercalated region of the amygdala also revealed lower levels of MOR (Vuong, Van Uum et al. 2010, Bodnar 2012). In contrast, KOR localizes heavily in the PVN and median eminence of the hypothalamus with no MOR detectible in these areas (Vuong, Van Uum et al. 2010). The high density of KOR together with a high concentration of dynorphin, an endogenous KOR agonist, in the PVN suggests that KOR and its ligand are closely involved in the stress response (Szeto 2003). Chronic activation with prenatal exposure to each opioid receptor agonist during the developmental period could potentially lead to different changes and functional adaptation of the HPA-axis and the brain areas where it locates. However, this speculation requires further studies. Although previous data demonstrate that chronic exposure to opioids can affect the HPA-axis circuitry—for example, chronic exposure to morphine increases CRF content in the hypothalamus of adult male rats (Milanes, Laorden et al. 1997)—very little is known how chronic exposure to MOR or KOR agonists modifies the HPA-axis during its developmental period.
Although the results from our previous study also showed the same stimulatory effects of prenatal oxycodone exposure on the HPA-axis, only ACTH levels were increased after the CRH stimulation test in oxycodone-exposed male rats compared to CON males. On the other hand, in this experiment, only the CORT levels were higher in female oxycodone-exposed rats after the RST compared to CON females. The differences in the results could be due to many factors. First, we have used different species of rats in our models. In the previous experiment, we have used Dark-Agouti (DA) rats, while for this one we use SD rats. It has been shown by multiple previous studies that different strains of rats, or even the same strain of rat from different vendors, respond to the same stress paradigm differently, as determined by various measures including CORT levels (Pecoraro, Ginsberg et al. 2006, Marissal-Arvy, Gaumont et al. 2007, Mozhui, Karlsson et al. 2010) The DA rat was used in the previous experiment because it may be a closer model to the human than the SD rat because the DA rat is genetically deficient in the CYP2D1 enzyme (Barham, Lennard et al. 1994), as described in the Introduction. A subsequent study, however, demonstrated that both DA and SD rats are suitable models for studying oxycodone’s pharmacology. Therefore, in this experiment we chose to use more available SD rats. Still, DA rats had consistently higher exposure to oxycodone and noroxycodone (κ-opioid agonist) than SD rats after oxycodone administration (Huang, Edwards et al. 2005). Thus this could in part lead to the different results.

Secondly, we have used different modalities to assess the HPA-axis function. RST was used in this study, whereas we had previously used CRH stimulation (Chapter 3). CRH can directly and potently stimulate the pituitary gland to secrete ACTH in vivo (Rivier, Brownstein et al. 1982) and in vitro (Vale, Spiess et al. 1981), bypassing the hypothalamic and higher control of the HPA-axis, such as the hippocampus. In addition,
the ACTH response to CRH is dose-dependent. Intravenous administration of the high dose of CRH (10 µg/kg) that we used would stimulate an enormous ACTH response from the pituitary gland (Mazzocchi, Rebuffat et al. 1989). Such a strong stimulation of the pituitary gland by hypothalamic CRH likely does not occur during stimulation of the HPA-axis by other stressors, such as restraint stress, which requires the response of the PVN itself. The fact that the HPA-axis response during restraint stress involves the activation of the PVN is supported by the finding of increased Fos expression in the parvocellular part of the PVN, mostly in the region that contains the CRH neurons (Girotti, Weinberg et al. 2007, Motta and Canteras 2015). There is also an increase in CRH mRNA expression (Kalin, Takahashi et al. 1994). Higher limbic structures, including the hippocampus, closely regulate the HPA-axis response to RST (Sapolsky, Krey et al. 1984, Herman, Dolgas et al. 1998, Herman, Ostrander et al. 2005). Although the hippocampus can either inhibit or stimulate the HPA-axis in a stress-specific manner, the hippocampus plays an important inhibitory role on the HPA-axis response to restraint stress (Herman, Ostrander et al. 2005). This is supported by previously published data indicating that destruction of all or part of the hippocampus increases HPA-axis response during restraint stress (Herman, Cullinan et al. 1995, Herman, Dolgas et al. 1998). Therefore the ACTH response during restraint stress may not be as exaggerated as occurred after administration of CRH due to the inhibitory regulation of the hippocampus.

The dissociation of ACTH from glucocorticoids has been more apparent in recent years and it is of clinical relevance. Several studies demonstrate that opioids, growth factors, and neuropeptides are capable of modulating the glucocorticoid response independent of ACTH (Bornstein, Engeland et al. 2008). We found that prenatal oxycodone exposure increases the CORT response to restraint stress in female rats.
without the expected increase in ACTH. This may be explained by the fact that CORT levels and adrenal responsiveness are not solely controlled by ACTH, but also by other systems including the sympathoadrenal-axis, which could have been affected by prenatal oxycodone exposure. In addition to hormonal control, the adrenal cortex is also regulated by extensive cholinergic preganglionic and catecholaminergic postganglionic sympathetic innervation and sensory nerves (Ehrhart-Bornstein, Hinson et al. 1998, Engeland, Ennen et al. 2005). The role of sympathetic control of adrenal cortical responsiveness is supported by many previous studies. For example, sympathetic nerve activation stimulates the release of cortisol (Bornstein, Ehrhart-Bornstein et al. 1990). Sympathectomy suppressed the maternal separation-induced increase in adrenal sensitivity to ACTH (Walker 1995), reduced corticosteroid responses to hypoxia in neonatal rats (Raff, Lee et al. 2004), and also reduced water deprivation-induced increases in plasma CORT in adult rats (Ulrich-Lai and Engeland 2002). Although in this experiment we did not directly investigate the effects of prenatal oxycodone exposure on sympathoadrenal-axis activity, it has been shown that prenatal exposure to opiates induces alterations in the sympathetic system in sex-specific manner. Previous studies demonstrated that prenatal exposure to opiates, such as morphine, increase hypothalamic norepinephrine (NE) content and turnover rate in the male but decrease hypothalamic NE content in the female rats (Vathy and Katay 1992). Prenatal exposure to morphine also altered the immunoreactivity of the rate-limiting enzyme in catecholamine biosynthesis, tyrosine hydroxylase (TH), in the locus coeruleus (LC) and the PVN, which are stress-sensitive brain regions, in a sex-specific manner; prenatal morphine exposure increased the density of TH-immunoreactivity (IR) in the male rats, but decreased the density of TH-IR in female rats that were ovariectomized (Vathy 1999). In addition, we found that POE is associated with a slightly larger
increase in conditional blood pressure during stress, suggesting that POE has effects on sympathetic control (Sithisarn, Bada et al. 2013) and Chapter 6.

Interestingly, perinatal exposure to 0.5 mg/kg/day of oxycodone but not to the higher dose of 2.0 mg/kg/day increased the CORT response to restraint stress in the female offspring. This may be due to development of tolerance in rat dams that received the higher dose of oxycodone, which includes tolerance to the stimulatory effects of oxycodone on the HPA axis. Opioid tolerance is characterized by decreased opioid effects on many pharmacologic and physiologic processes, which include analgesia, euphoria, sedation, etc. (Dumas and Pollack 2008). Tolerance to the antinociceptive effects of opioid agonists has been extensively studied and well described to be pharmacodynamic, time- and dose-dependent, and receptor-specific (Collett 1998). Opioid tolerance to the modulatory effects on the HPA has also been described to be receptor-specific (Iyengar, Kim et al. 1987, Ignar and Kuhn 1990). Iyengar et al demonstrated that although acute administration of the KOR agonists, dynorphin and MEAP, dose-dependently and potently stimulated the release of CORT in SD rats, these effects were abolished when rats were previously made tolerant to dynorphin and MEAP by chronic exposure to a different KOR agonist, U 50488H (Iyengar, Kim et al. 1987). In addition, Ignar et al have shown that after chronic exposure to intra-peritoneal injection of escalating doses of U 504,88H for five days (1mg/kg increasing 1 mg/day to 5 mg/day), rats developed tolerance to the acute stimulatory effect of U 504,88H on CORT secretion (Ignar and Kuhn 1990). It is not well studied whether or not tolerance to the stimulatory effects on the HPA-axis of KOR agonists is dose-dependent; tolerance to the MOR agonist, however, has been shown to be dose-related. Chronic administration of morphine at a lower dose (0.5 mg/100 g body weight i.p daily) for seven days had no significant effects on plasma and adenohypophysis ACTH concentrations and
hypothalamic CRH content, whereas a higher dose (2 mg/kg) caused a significant decrease in these parameters, suggesting a dose-related effect (el Daly 1996). Taken together, it is possible that the rat dams that were exposed to a higher dose of oxycodone (2 mg/kg/day) may have developed tolerance leading to a decreased fetal CORT exposure. This may have had different influences on the developing HPA-axis of the offspring such that CORT release during the RST was unaffected.

It appears in this study that perinatal oxycodone exposure produces different effects on the HPA-axis in male and female offspring, namely, elevated CORT levels in only female OXY. This sex-specific consequence is consistent with what has been described by us and others after perinatal drug exposure and stress paradigms (Bhatnagar, Lee et al. 2005, Weinstock 2007, Sithisarn, Bada et al. 2008, Grundwald and Brunton 2015). The sex disparity has been postulated to be due to different influences of sex hormone actions, particularly androgens, on male and female brain development during a critical period. These sex-specific differences include structure volumes, cell numbers, synaptic connections and neuronal morphology (Schwarz and McCarthy 2008, Charil, Laplante et al. 2010). Moreover, the activity and sensitivity of placental 11β-HSD2, the glucocorticoid regulating enzyme, which catalyzes the conversion of cortisol to its biologically inactive metabolite cortisone, appears to be sex-linked in both animals and humans (Braun, Li et al. 2009, Mina, Raikkonen et al. 2015, Penailillo, Guajardo et al. 2015). Therefore, gender interacts with placental 11β-HSD2 in regulating fetal exposure to CORT, and potentially impacts the long-term effect of POE on the development of the HPA-axis. It is also interesting that development of tolerance to the high dose of oxycodone in the dam affected only the female offspring. Whether this tolerance is related to the altered activity of the placental 11β-HSD2, which leads to gender differences on the development of the HPA-axis remains to be further elucidated.
The enhanced HPA-axis response to stress after POE in our study is consistent with previous findings that showed a heightened HPA-axis response to stress in the offspring after perinatal exposure to stress, when the rat dams were exposed to different paradigms of stress, such as unpredictable exposure to light and noise or social defeat (Weinstock, Matlina et al. 1992, Brunton and Russell 2010). The mechanisms behind HPA-axis dysregulation in perinatal stress models appear to involve changes in both excitatory feedforward and inhibitory feedback mechanisms (Seckl and Meaney 2004, Mueller and Bale 2008, Brunton 2015). We have further explored the mechanisms by which POE affects the HPA-axis and circuitry control, as will be described in detail in Chapter 5. We speculate that perinatal oxycodone exposure may alter 1) the expression of the CRH in the hypothalamus and limbic structures and 2) the feedback mechanisms of the HPA-axis, which involve the actions of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in the PVN, and the regulatory control of the HPA-axis by the limbic structures.

We encountered some limitations in this experiment. Daily injections through the subcutaneous port during pregnancy can add stress to the dams, which may affect the outcomes. We attempted to control for that by administering NS injections to the control dams as well. We plan to modify the treatment protocol by using a mini-pump to deliver treatment drugs in the future. In addition, we did not monitor estrous cycles of the female offspring at the time of RST; it has been previously shown that CORT response during RST is impacted by estrous cycles (Ariza Traslavina, de Oliveira et al. 2014).
Virgin SD rats

Right Atrial Cannulation one week before mating
Mating
Oxycodone 0.5 or 2 mg/kg or NS Injection daily GD 8-21

Oxycodone Postnatal Day (PD) 1, 3, & 5
Foster the pups PD 5

Foster Mom

PD 60-65: Restraint Stress Test
ACTH/CORT by RIA

Figure 4.1: Perinatal oxycodone treatment model and postnatal restraint stress test. Red font indicates modifications from previous model (figure 3.1)
Figure 4.2: Plasma ACTH concentrations (mean ± SEM) during and after restraint stress test (RST) in male (left panel) and female (right panel) rats treated at time 0 with NSS (CON, closed circles), a low dose of oxycodone (OXY-L, open circles), or a high dose of oxycodone (OXY-H, closed triangles). Litter numbers in the males: CON = 11, OXY-L = 5, OXY-H = 11; litter numbers in the females: CON = 12, OXY-L = 5, OXY-H = 12.
Figure 4.3: Plasma corticosterone (CORT) concentrations (mean ± SEM) during and after restraint stress test (RST) in male (left panel) and female (right panel) rats treated with NSS (CON, closed circles), low-dose oxycodone (OXY-L, open circles), or high-dose oxycodone (OXY-H, closed triangles). Litter numbers, males: CON=11, OXY-L=5, OXY-H=11; litter numbers, females: CON=12, OXY-L=5, OXY-H=12. The asterisks (*) indicate mean differences between the OXY-L and OXY-H groups (p<0.05);
5.1 Introduction

In Chapters 3 and 4, we have demonstrated that perinatal oxycodone exposure (POE) is associated with an enhanced HPA-axis response to stress as measured by an increased ACTH response in the males after CRH stimulation test and an increased CORT response in the females after restraint stress test. Potential mechanisms whereby POE can influence fetal HPA-axis development and programming may involve exaggerated stimulatory controls or impaired negative feedback mechanisms. The HPA-axis stimulatory response is controlled by the neurons in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN) which synthesize and secrete corticotropin releasing hormone (CRH) (Antoni 1986) (see Chapter 1). CRH stimulates ACTH release from the anterior pituitary corticotropes into the systemic circulation (Kovacs 2013). The binding of ACTH to MC2 receptors in the Zona fasciculata of the adrenal cortex stimulates the synthesis and release of glucocorticoid (Whitnall 1993). CRH neurons in the PVN initiate an HPA response to stress receive regulatory modulation from higher brain centers, including the limbic system, especially the CA1 and CA3 areas of the hippocampus, central nucleus of amygdala (CEA) and medial prefrontal cortex (PFC) (Herman, Ostrander et al. 2005). Although these limbic sites have no direct connection with the PVN, their efferents relay with neurons in the bed nucleus of the stria terminalis, and hypothalamus to access CRH neurons in the PVN (Herman, Ostrander et al. 2005). The regulation of the limbic system on the HPA-axis appears to be both region and stressor-specific, namely, the hippocampus exerts more...
of the inhibitory roles while the input from the amygdala activates the HPA-axis (Herman, Ostrander et al. 2005). CRH is also widely distributed in these limbic structures and its expression modulates a variety of stress responses. CRH expression is also affected by exposure to stress and corticosterone (Kovacs 2013). In utero milieus such as undernutrition and prenatal stress induce overexpression of CRH mRNA, CRH protein and subsequent CORT levels (Nunez, Ruiz et al. 2008, Garcia-Caceres, Lagunas et al. 2010). However, data are still lacking regarding the effects of prenatal exposure to opiates or oxycodone on the expression of CRH in the PVN and limbic system. Therefore in this chapter we determine the density of CRH neurons in the PVN by immunocytochemistry (ICC) and also the expression of CRH m-RNA in the PVN and limbic systems by quantitative real time polymerase chain reaction (QRT-PCR). We hypothesize that POE is associated with an increase in CRH neuron counts in the PVN and/or CRH mRNA expression in the PVN, and possibly in the limbic structures.

We found sex-differences in the results when the males, prenatally exposed to oxycodone, had a significantly elevated ACTH response compared to the controls after the CRH stimulation test; this heightened response was not observed in the females (Chapter 3). On the other hand, an enhanced CORT response to the restraint stress test only occurred in the females prenatally exposed to oxycodone, not the males (Chapter 4). Sex differences in the HPA function are partly due to the modulatory effects of the gonadal hormones (Viau and Meaney 1991, Lund, Munson et al. 2004). Female rats exhibit a more robust HPA response to stress compared to male rats (Handa, Burgess et al. 1994). It appears that in the male, androgen inhibits whereas in the female, estrogens function to enhance the activity of the HPA-axis (Handa, Burgess et al. 1994, Viau and Meaney 1996, Lund, Munson et al. 2004). It is noteworthy that both male and female gonadal hormones can modulate the HPA-axis through the estrogen receptor beta.
(ERbeta) (Miller, Suzuki et al. 2004, Lund, Hinds et al. 2006). Testosterone can thus exert its inhibitory action at ERs in those tissues that express the aromatase enzyme, which converts it to estradiol. But the action of testosterone in inhibitory modulation of the HPA-axis may also involve its 5α-reduction within the central nervous system to dihydrotestosterone (DHT) (Handa, Kudwa et al. 2013). DHT cannot be aromatized to estrogen and therefore, acts as a pure androgen on androgen receptor (AR) (Swerdloff and Wang 1998), suggesting that AR may also be involved in modulating the function of the HPA-axis, but through several receptor-signaling pathways (Handa, Kudwa et al. 2013) as ARs are not found in the medial parvocellular neurons of the PVN (Bingham, Williamson et al. 2006). In this experiment, we will focus on ERbeta, which is found in relatively high levels in the PVN (Hrabovszky, Kallo et al. 1998, Somponpun and Sladek 2003) and expressed within the CRH neuron (Miller, Suzuki et al. 2004) while the expression of another subtype of ER, ERalphaERalphaERalphaERalphaERalpha, is scarce in the PVN (Shughrue, Lane et al. 1997). The role of ERbeta is to integrate the modulatory effects of gonadal hormones on the HPA-axis through CRH-dependent mechanisms in an inhibitory manner for androgen (Lund, Hinds et al. 2006) and a stimulatory manner for estrogens (Miller, Suzuki et al. 2004, Chen, Zhu et al. 2008). Thus, we aim to study the subpopulation of the CRH neurons in the PVN that are immunopositive for ERbeta and also analyze ERbeta and ERalphaERalphaERalphaERalphaERalpha mRNA-expression in the PVN and the limbic system by QRT-PCR. We hypothesize that POE is associated with an increase in CRH neurons in the PVN that are also immunopositive for ERbeta, and/or there is an increase in ERbeta mRNA expression in the PVN or in the limbic structures. These changes may lead to enhanced ACTH and CORT responses to the stress test in the offspring prenatally exposed to oxycodone.
Another possible pathway whereby POE may lead to the enhanced HPA-axis response to stress is through the impairment in negative feedback mechanisms. The feedback processes occur by the action of the corticosteroids at the mineralocorticoid receptor (MR) and/or glucocorticoid receptors (GR) (de Kloet 2014). These two related nuclear receptors are located at multiple sites of the HPA-axis including in the PVN, the pituitary gland, and the limbic system. The feedback mechanisms functionally vary according to the type, location, and intensity of the stressors (i.e. acute vs chronic, physical vs psychological) and the neuroanatomical pathways responsible for each stress (Keller-Wood 2015). The balance between MR and GR actions is also important; it has been proposed that the imbalance between MR and GR-mediated actions in the limbic system may impair the functions of the HPA-axis. This impairment can potentially lead to vulnerability or resilience to affective diseases (De Kloet, Vreugdenhil et al. 1998, Harris and Seckl 2011). Early life events such as prenatal stress and antenatal exposure to glucocorticoids have been shown to increase HPA-axis reactivity. This could be possible through relatively inadequate GR and MR negative feedback sensitivity related to reduced GR and MR expression in the hippocampus and other sites (Noorlander, De Graan et al. 2006, Shoener, Baig et al. 2006, Mueller and Bale 2008). Therefore we hypothesize that POE results in decreases in GR and/or MR expression in the PVN and limbic system, resulting in the enhanced HPA-axis response to stress. We analyzed MR- and GR-mRNA expression in the PVN and the limbic areas by QRT-PCR as described below.
5.2 Material and Methods

5.2.1 Experimental Design: Animals and Prenatal Treatments

The animals received pre- and peri-natal treatment as described in Chapter 4. For the purpose of this current experiment, only the offspring that received high doses of oxycodone (OXY, 2.0 mg/kg/day, number of dams = 5) or an equivalent volume of vehicle (CON, NSS 1.0 ml/kg/day, number of dams = 5) from GD 8-21 were included. Neonatal rats were counted and weighed. Litters were adjusted to contain equal numbers and a balanced proportion of male and female pups when possible. Rat pups were transferred to the care of foster dams on PD 5. The pups were weighed daily and weaned at PD 25 when they were separated by sex. They were randomly selected for RST on PD 50-60 and a few hours after they completed the RST, all were perfused intracardially that afternoon, and their brains were removed and processed for immunocytochemical co-localization of ERbeta and CRH as described below. Litter mates of the offspring of both sexes from each litter that were not subjected to RST, were randomly selected and terminated on PD 60-70 to harvest brains for analysis of MR and GR mRNA by polymerase chain reaction as described below.

5.2.2 Brain Perfusion

Rat brains were perfused as described in Chapter 2 and stored in cryoprotectant at -20°C until processed by dual label immunocytochemistry.

5.2.3 Dual-label Immunocytochemistry (ICC)

The ICC procedure was performed by means of a method previously described by Legan & Tsai (Legan and Tsai 2003). Twenty to twenty four selected sections of the PVN (corresponding to Bregma -1.4 to Bregma -1.8, (Paxinos 1998)) were first rinsed in 0.05 M potassium phosphate buffered saline (KPBS) for six minutes each for ten
times, then placed in freshly made 1% sodium borohydrate for 20 minutes at room
temperature. The sections were rinsed several times in 30 minutes to remove the
bubbles. The sections were then incubated in rabbit anti-human ERbeta, dilution 1:1000
(Zymed) in 10% normal goat serum (NGS) in 0.4% Triton X-100 (TTX)-KPBS for one
hour at room temperature followed by 72 hours at 4º C. Sections were then rinsed 10
times over one hour then incubated for 1 hour at room temperature in 1-2 ml of 1:600
biotinylated goat anti-rabbit IgG in 0.4% TTX-KPBS. Sections were rinsed 5 times, 10
minutes each in KPBS then incubated in A/B solution made with ABC Elite Kit (Vector)
using 45 µl of A and 45 µl of B per 10 ml of KPBS with 0.4% TTX-KPBS for 1 hour at
room temperature. To visualize the ERbeta, sections were rinsed 3 times, 5 minutes
each with KPBS then 3 times, 5 minutes each with 0.175 M sodium acetate (pH=6.5)
then reacted in 1-2 ml of nickel-enhanced 3, 3’-diaminobenzidine tetrahydrochloride
(DAB) solution (2 mg DAB plus 250 mg Nickel (II) Sulfate with 8.3 µl 3% H2O2 per 10 ml
of 0.175 M sodium acetate) to yield a black reaction product. Sections were rinsed with
KPBS, 5 minutes each for 3 times then incubated with rabbit anti-CRH (courtesy of Dr.
Ann-Judith Silverman), dilution 1:50,000 in 10% NGS in 0.4% TTX-KPBS as a second
immunolabeling at 4º C for 48 hours. The procedure as described above was followed
through the incubation with A/B solution. Sections were rinsed 3 times, 5 minutes each
in KPBS then 3 times, 5 minutes each in TBS (Trizma base, pH7.2, 0.769 g Tris per 100
ml saline). Sections were then reacted in DAB chromogen solution (0.2 ml (2 mg) 1%
DAB (freshly made with 0.1 g/10 ml TBS) with 8.3 µl 3% H2O2 per 10 ml of TBS) to yield
a brown reaction product for about 15 minutes. After the chromogen reactions had
developed, sections were rinsed 3 times, 5 minutes each in TBS then 3 times, 5 minutes
each in KPBS before mounting on gelatin-coated slides.
5.2.4 Image Analysis of CRH- and ERbeta Immunopositive Neurons

All sections were examined under a light microscope (Eclipse E600, Nikon). CRH and ERbeta immunopositive neurons from both left and right hemispheres were identified in the PVN (Figure 5.1a, Bregma -1.8). The numbers of CRH neurons that were ERbeta-immunopositive or not and the numbers of non-CRH neurons that were immunopositive for ERbeta were manually counted by an observer blind to the treatment groups by visualization at a magnification of 200-400X (Figure 5.1b). The entire area of the PVN was examined under a grid and neurons were counted in each subdivision serially to ensure that the neurons were counted only once. Only neurons containing nuclei were counted. ERbeta staining was identified as a black product in the nucleus and CRH staining was identified by a brown chromogen product in the cytoplasm. ERbeta/CRH neurons were counted when a black product was identified in the nucleus and a brown chromogen product could be identified in the cytoplasm of the same cell. ERbeta immunonegative/CRH neurons were identified when a brown chromogen product could be identified in the cytoplasm with an empty nucleus in the same cell (Figure 5.1c). The observer was blind to the treatment groups during image analysis. Numbers of CRH- and ERbeta immunopositive neurons were averaged from 3 comparable sections of both sides of the PVN of each rat for further statistical analysis.

5.2.5 Brain Tissue Collection and RNA Isolation

Offspring were terminated for brain harvest at PD 60-70. The female rats were at random stages of the estrous cycle. Micro-punches of brain tissue were removed for analysis of MR and GR mRNA using sterile biopsy punches 1 and 2 mm in diameter. In each animal, tissue punches were obtained from the following 5 brain areas that are involved in HPA-axis response to stress: 1) prefrontal cortex (PFC) (approximately Bregma +3.5 to Bregma 2.7), 2) PVN (approximately Bregma -1.3 to Bregma -1.8), 3)
central nucleus of amygdala (CEA) (approximately Bregma -1.8 to Bregma -2.5), 4) hippocampal CA1 (approximately Bregma -3 to Bregma -4.2) and 5) hippocampal CA3 (ventral part) (approximately Bregma -4.2 to Bregma -4.6) according to Paxinos & Watson (Paxinos 1998).

RNA was isolated using a modified guanidine isothiocyanate (GIT) method (Chomczynski and Sacchi 1987). Brain tissue punches were homogenized in 250 µl GIT buffer (4 M guanidine thiocyanate, 1 M sodium citrate (25 mM final), 10% sarcocyl (0.5% final), 0.1 M β-mercaptoethanol and diethylpyrocarbonate (DEPC)-treated water (DEPC water). After incubation for 5 minutes on ice, 30 µl 2 M sodium acetate (NaOAc), 300 µl of phenol and 60 µl of chloroform/isoamyl alcohol were added serially with mixing after each addition. The mixture was incubated on ice for 15 minutes, centrifuged at 13,000 rpm for 10 minutes at 4º C and the aqueous phase was transferred to a new tube. After adding 200 µl of isopropyl alcohol to the tube and mixing, the tube was then placed on dry ice for at least 30 minutes until the mixture became solid then centrifuged at 13,000 rpm for 10 minutes at 4º C. The supernatant was aspirated and the pellet was re-suspended in 100 µl GIT buffer. Then 10 µl 3 M NaOAc, 100 µl isopropyl alcohol were added and mixed. The reaction mix was placed on dry ice until solid then centrifuged at 13,000 rpm for at least 30 minutes at 4º C until a pellet formed. Supernatant was then poured off without disturbing the pellet and the pellet was washed with 500 µl of 70% ethanol. The mixture was again centrifuged at 13,000 rpm for 30 minutes at 4º C. The ethanol was poured off and the pellet was allowed to dry open for at least 30 minutes. The pellet of RNA was re-suspended in 30 µl of RNase-free water (DEPC-water). Five µl of isolated RNA was added to 95 µl DEPC water for determination of nucleic acid purity by UV spectrophotometry (Photometer WPA Biotech UV1101®) with an acceptable
A260/A280 ratio of close to 2. The suspended RNA was stored at −80° C until reverse transcription.

5.2.6 Reverse Transcription of RNA (making cDNA)

One µg of suspended RNA was mixed with 1 µl of Random Primers (Invitrogen) and 1 µl of 10 mM deoxynucleotides (dNTPs). DEPC water was added to achieve a volume of 12 µl for each reaction. The samples were incubated at 65° C for 5 min and then chilled on ice, and centrifuged briefly. Four µl of 5X first strand buffer, 2 µl 0.1 M dithiothreitol (DTT), 1 µl ribonuclease inhibitors (RNasin), and 1 µl superscript RT (Invitrogen) were added to each sample, and mixed well by gently pipetting up and down. Samples were then incubated at room temperature for 10 min, 42° C for 50 min, and 70° C for 15 min. Samples were stored at -80° C until quantitative real time polymerase chain reaction (QRT-PCR)

5.2.7 Quantitative Real-time Polymerase Chain Reaction (QRT- PCR)

The method was modified from a previously published study (Prewitt and Wilson 2007). For QRT-PCR, each reaction contained 10.125 µl of DEPC water, 12.5 µl of 2X SYBRGreen Brilliant Master Mix (Stratagene, La Jolla, CA), 0.5 µl of forward primer (at a concentration of 250 nM for CRH, ERbeta, ERalphaERalphaERalphaERalpha, GR, Histone; 125 nM for MR), 0.5 µl of reverse primer (at a concentration of 50 nM for CRH, ERbeta, ERalphaERalphaERalphaERalpha, GR, Histone; 125 nM for MR), 0.375 µl of reference dye (Stratagene, diluted 1:500) and 1 µl of appropriate cDNA of interest. Each 96 well plate contained a non-template control and each sample was run in triplicate. The PCR product was run on 2% agarose gel electrophoresis to check the specific amplification. Cycling parameters were as follows: 1 cycle at 95° C for 10 min, 40 cycles of 95° C for 30 s, annealing temperature for 1 min, 72° C for 30 s, and 1 cycle of 95° C for 1 min and 55° C for 30 s. Real time fluorescent measurements were taken at every
cycle and change in threshold cycle (ΔCt) was calculated. All data were normalized to
the housekeeping gene Histone 3.1. Primers used to amplify ERbeta, ERalpha and
Histone 3.1 have been previously described (Kuiper, Carlsson et al. 1997, Prewitt and
Wilson 2007). Sequences of amplifier primers are listed in table 5.1

5.2.8 Statistical Analyses
We compared the effects of treatments within each sex using One Way Analysis
of Variance (ANOVA). Results were considered significant at p < 0.05. We analyzed the
results from each sex separately because it is well known that males and females have
different ACTH and CORT responses to stress (McCormick, Smythe et al. 1995, Nock,
Cicero et al. 1998, Koehl, Darnaudery et al. 1999) which is consistent with the analysis
in Chapter 4. Dependent variables from the ICC experiment include total numbers of
CRH neurons, numbers and percent of total ERbeta positive/CRH neurons, numbers
and percent of total ERbeta negative/CRH neurons and numbers of ERbeta
positive/CRH negative cells. Dependent variables from RT-PCR experiments include
CRH, ERbeta, ERalphaERalphaERalphaERalphaERalphaERalphaERalphaERalphaMR, and GR gene expression in the
PVN, CA1 and CA3 areas of the hippocampus, CEA and PFC, calculated in fold change
over control.

5.3 Results
5.3.1 The Effects of POE on Immunopositive CRH and ER-β in the PVN
POE had no effects on the numbers of total immunopositive CRH neurons in
OXY and CON groups in either male (OXY 115.4 (± 18.7) vs CON 143.1 (± 5.6), p =
0.19) (Figure 5.2a, left panel) or female rats (OXY 152.3 (± 8.7) vs CON 182.0 (± 20.7),
p = 0.27) (Figure 5.2a, right panel).
Although POE had no effect on the numbers of ERbeta immunopositive CRH neurons in either male (OXY 83.3 (± 17.5) vs CON 68.7 (± 4.5), p = 0.44) or female rats (OXY 91.9 (± 3.1) vs 71.1 (± 7.9), p = 0.06) (Figure 5.2b), in utero OXY exposure increased the proportion of total CRH neurons that were ERbeta immunopositive in both males and females (Figure 5.2c). In male OXY rats, 70.1 (± 5.8) % of total CRH neurons were immunopositive for ERbeta as compared to 48.2 (± 3.1) % in male CON rats (p = 0.018) (Figure 5.2c, left panel). OXY female rats also had a higher percentage of ERbeta immunopositive/CRH neurons as compared to female CON rats (60.6 (±1.5) % vs 39.4 (±1.7) %, p <0.001) (Figure 5.2c, right panel).

POE lowered the number of ERbeta immunonegative CRH neurons in both male and female OXY rats compared to CON rats of the same sex (Figure 5.3a). In male rats, the OXY group had smaller numbers of ERbeta immunonegative CRH neurons compared to the CON group (32 (± 6.6) vs 74 (± 4.8), p<0.001, figure 5.3a left panel). In female rats, the OXY group also had lower numbers of ERbeta immunonegative CRH neurons compared to the CON group (60 (± 5.8) vs 111 (± 13.8), p = 0.018, figure 5.3a right panel).

Similarly, the percentages of ERbeta immunonegative CRH neurons of the total CRH neurons were also significantly lower in OXY rats, both in the males (OXY 29.9 (± 5.8) vs CON 51.9 (± 2.4), p =0.008) (Figure 5.3b, left panel) and in the females (OXY 39.4 (± 1.5) vs CON 60.6 (± 1.7), p<0.001) (Figure 5.3b, right panel).

The numbers of ERbeta positive/CRH negative neurons in the PVN were not different between treatment groups in either male (OXY177 (± 18.2) vs CON 154 (± 3.6), p = 0.25) or female rats (132 (± 16.9) vs 138 (± 19.6), p= 0.84) (Figure 5.4). There were more ERbeta positive neurons than ERbeta positive CRH neurons in both treatment
groups and sexes suggesting that there were non-CRH neurons in the PVN areas that also expressed ERbeta. These neurons with larger nuclei may include the population of vasopressin-expressing neurons in the parvocellular and magnocellular parts and oxytocin containing neurons in the medial parvocellular part of the PVN based on the anatomical locations in the sections and previously published data by Suzuki et al and Weiser et al (Suzuki and Handa 2005, Weiser, Foradori et al. 2008).

5.3.2 Quantitative Real-time Polymerase Chain Reaction (QRT-PCR) Analysis of Gene Expression in Stress Circuitry

5.3.2.1 In the PVN

POE did not change CRH mRNA expression in the male rats but in the female, OXY rats had a trend toward decreased CRH-mRNA expression compared to CON (p=0.055) (Figure 5.5a). There were no differences between treatment groups in ERbeta-, ERalphaERalphaERalphaERalpha-, or GR mRNA-expression in both male and female rats (Figure 5.5b, 5.5c and 5.6a respectively). Interestingly, POE increased MR-mRNA expression in OXY male rats (1.67 fold) compared to male CON rats (p=0.022) (Figure 5.6b, left panel). This elevation of MR-expression was not observed in the female OXY compared to female CON.

5.3.2.2 In the CA1 Area of Hippocampus

There were no differences in mRNA expression in all genes tested, including CRH, ERbeta, ERalphaERalphaERalphaERalpha, GR and MR in both male (Figure 5.7a) and female rats (Figure 5.7b).
5.3.2.3 In the CA3 Area of Hippocampus

CRH-, ERbeta-, ERalphaERalphaERalphaERalpha and GR-mRNA expressions were not different between treatment groups and sexes (Figure 5.8a, 5.8b, 5.8c and 5.9a respectively). MR mRNA expressions were not different between treatment groups in male rats (Figure 5.9b, left panel). However, POE significantly decreased MR-mRNA expression in OXY female rats compared to CON females in the CA3 area (p=0.017) (Figure 5.9b, right panel).

5.3.2.4 In the Central Nucleus of the Amygdala (CEA)

POE significantly decreased CRH-mRNA expression in female OXY compared to CON female rats (p=0.003) (Figure 5.10a, right panel); this decrease in CRH mRNA expression was not observed in male OXY. In the males, there was no significant difference in CRH-mRNA expression between treatment groups; this could be due to the wide SEM (Figure 5.10a, left panel). There were no differences in mRNA expression of the other genes tested, including ERbeta, ERalphaERalphaERalphaERalpha, GR and MR in either males or females (Figures 5.10b, 5.10c, 5.11a, and 5.11b respectively).

5.3.2.5 In the Prefrontal Cortex (PFC)

There were no differences in mRNA expression in all genes tested, including CRH, ERbeta, ERalphaERalphaERalphaERalpha, GR and MR in both male (Figure 5.12a) and female rats (Figure 5.12b).
5.4 Discussion

In this experiment we explored the effects of perinatal oxycodone exposure on the expression of receptors mediating feedback control of the HPA-axis, which may partly explain the mechanisms by which POE enhanced the response to stress of the HPA-axis, as demonstrated in Chapters 3 and 4. The results were summarized in Figure 5.13. The changes may involve both increased excitatory and/or impaired inhibitory feedback mechanisms at different levels of the control of the HPA-axis, including the paraventricular nucleus (PVN) and the limbic system which includes the CA1 and CA3 areas of the hippocampus, the central nucleus of amygdala (CEA) and the prefrontal cortex (PFC). We hypothesized that the enhanced HPA-axis response to stress after POE may result from increased excitatory regulation of the HPA axis, which includes an increase in CRH immunoreactive neurons and CRH-mRNA expression; POE may also reduce the inhibitory modulation of the HPA-axis by decreasing negative feedback mechanisms, which would include decreases in GR and MR expression, or a combination of these adaptations.

In the PVN, we did not find significant increases in total CRH neuron counts or CRH-mRNA expression as hypothesized. We compared our results with previous studies on the effects of prenatal morphine and opiate exposure, prenatal stress exposure and chronic opiate exposure on CRH. There are actually limited published data on the effects on prenatal morphine or other opiates on CRH m-RNA expression or CRH content in the PVN. One previous study by Dutriez-Casteloot et al, found no changes in the hypothalamic CRF content under resting conditions in adult male rats prenatally exposed to morphine (Dutriez-Casteloot, Bernet et al. 1999). The exposure to prenatal stress, when compared to other stress paradigms, more consistently resulted in increased CRH-mRNA expression in female rats (Garcia-Caceres, Lagunas et al. 2010,
Zohar and Weinstock 2011), while the effects of chronic opioid exposure in adult rats on CRH is more conflicting (Vuong, Van Uum et al. 2010). Although acute morphine administration robustly increased CORT (Jezova, Vigas et al. 1982), ACTH (Jezova, Vigas et al. 1982, el Daly 1996) and hypothalamic CRH content in response to stress in adult male albino rats (el Daly 1996), the effect of chronic morphine exposure on the HPA-axis responsiveness depended on the dose, time course and the duration after withdrawal of the drugs (Vuong, Van Uum et al. 2010). El Daly demonstrated that postnatal chronic administration with morphine (0.5 mg/100 g body weight intraperitoneally) daily for seven days had no effect on hypothalamic CRH content in adult rats. However, when the dose was increased to 2 mg/100 g body weight, hypothalamic CRH content was significantly decreased (el Daly 1996). Buckingham et al had also shown that in contrast to enhanced secretory activity observed in the hypothalami removed from adult male rats treated acutely with morphine, the hypothalami of morphine-tolerant rats did not secrete CRF in response to morphine (Buckingham and Cooper 1984). Together with these findings, the trend of the down regulation of hypothalamic CRH-mRNA expression in OXY female rats in a resting condition in our experiment may represent the adaptation of the HPA axis after perinatal exposure to oxycodone, as is observed after chronic exposure to morphine. Alternatively, the response of the HPA-axis to oxycodone may be different from the response to morphine, or perinatal opiate treatment may affect the HPA-axis differently compared to post-natal treatment.

We found sex differences in the CORT response to the restraint stress test in Chapter 4, with increased CORT concentrations in female rats prenatally exposed to oxycodone, but not the males. Therefore, we analyzed the subpopulations of the CRH neurons in the PVN, whether they are ERbeta immunopositive or not and also studied
mRNA-expression of both ERbeta and -alpha in the PVN and in the limbic system. We did not find differences in either ERbeta or ERalpha mRNA expression between treatment groups in any brain areas. Interestingly, both male and female oxycodone exposed rats had significantly higher percentages of ERbeta immunopositive CRH neurons in the PVN compared to CON rats of the same sex. Likewise, the numbers and percentages of CRH neurons that were ERbeta immunonegative in the PVN of both male and female OXY offspring were significantly decreased compared to CON offspring of the same sex. These findings may in part explain the enhanced CORT response to the restraint stress test in the females prenatally exposed to oxycodone (Chapter 4). Because previous studies had shown that estradiol’s stimulatory effects on CRH gene expression require ERbeta (Miller, Suzuki et al. 2004, Chen, Zhu et al. 2008, Handa, Mani et al. 2012), we may postulate that in the females, the increase in the proportion of CRH neurons that co-localize with ERbeta may increase the stimulatory modulation of estrogens on the CRH in the PVN. This in turn may result in an enhanced HPA-axis response to a stress that requires activation of the PVN, such as the restraint stress test (RST). On the other hand, androgen had been shown to inhibit the HPA-axis response through ERbeta expressing neurons in the hypothalamus (Lund, Hinds et al. 2006). Therefore it is possible that the higher percentage of ERbeta positive-CRH neurons in the PVN of male POE rats may heighten the inhibitory modulation, resulting in no increase in the CORT response to RST as observed in the female. In addition, the ACTH levels in male OXY rats were heighten after the CRH stimulation test compared to male CON (Chapter 3). Although the mechanisms by which testosterone (T) acts to influence the HPA functions have not been clearly resolved, these may involve the action not only through ERbeta (Lund, Hinds et al. 2006) but also the 5α-reduction of T to DHT which acts through AR (Handa, Kudwa et al. 2013). Therefore, it is possible that POE may impair this inhibitory system
in the male by either decreasing testosterone, 5α-reduction to DHT or AR expression which in turn, leads to enhanced ACTH response to CRH stimulation. Studies in humans and rats support the possibility that exposure to opiate and/or oxycodone is associated with decreased testosterone levels in the males (Vodo, Arcelli et al. 2013, Eichenbaum, Gohler et al. 2015, Rubinstein and Carpenter 2016) but thus far, limited data are available in regards to how POE may affect the 5α-reduction of T to DHT or AR expression. Furthermore, we speculate that decreased levels of testosterone and/or estrogen may be one possible basis which lead to an increase in ERbeta immunoreactivity since it has been previously shown that chronic exposure to opiates reduces the levels of these gonadal hormones (Yilmaz, Konar et al. 1999, Vuong, Van Uum et al. 2010, 2012, Elliott, Opper et al. 2012) and changes in gonadal hormone levels site-specifically affect ERbeta expression (Nomura, Korach et al. 2003). Limited data are available regarding the effects of prenatal opiate exposure on the gonadal axis. Prenatal morphine exposure did not change gonadal activity in an early postnatal period (Lesage, Bernet et al. 1996), however, no data are available on how perinatal exposure to oxycodone affects the testosterone or estrogens levels; therefore these speculations warrant further investigations.

Compared to hypothalamic CRH, the expression, function and regulation of extrahypothalamic CRH, including in the CEA, has been much less studied (Kovacs 2013). It has been postulated that the regulatory role of the CEA on the HPA-axis appears to be, like other limbic regions, stressor-specific and, in contrast to other limbic regions, potentiating rather than inhibiting in HPA response to stress (Herman, Ostrander et al. 2005). Thus, based on the enhanced ACTH and CORT response to stress after POE as described in Chapters 3 and 4, we hypothesized that POE may result in increased CRH-mRNA expression in the CEA. However, we did not find the
expected increase but rather, a decrease in CRH-mRNA expression in the CEA of female oxycodone exposed rats, and a strong trend for a decrease in CRH-mRNA expression in the PVN of these rats. Our finding is in contrast to the results from perinatal stress paradigms, which reported an increase in CRH expression and content in the CEA (Cratty, Ward et al. 1995, Mueller and Bale 2008). Rats who underwent morphine withdrawal also had increased CRH-mRNA expression in both the PVN and the CEA with attenuation of the withdrawal symptoms after microinjection of CRH-antagonist to the CEA, suggesting the role of amygdala CRH in opiate dependence (McNally and Akil 2002). Nevertheless, available published data on how perinatal oxycodone exposure affects the CRH-mRNA expression in the CEA for comparison with our results is limited. The differences in the results could be due to different opiate agonist subtypes used (MOR vs KOR agonists), the route, dose, and timing of administration during brain development, among others as discussed in Chapter 4; these methodology issues make it difficult to compare the results from different studies.

Another possible mechanism by which POE enhances the HPA-axis response to stress is that it impairs negative feedback mechanisms. Glucocorticoid feedback action in the HPA-axis is mediated by glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) that are expressed in both the parvocellular and magnocellular regions of the PVN (Han, Ozawa et al. 2005, Chen, Gomez-Sanchez et al. 2014). The feedback control of the HPA-axis also involves indirect modulation of the PVN by the actions of corticosterone on these receptors in the limbic system including the hippocampus, CEA and PFC (Reul and de Kloet 1985, Ratka, Sutanto et al. 1989, Herman, Cullinan et al. 1992, De Kloet, Vreugdenhil et al. 1998, de Kloet 2014). From this present study, we did not find decreases in GR expression in any brain regions as hypothesized. However, we found that in the PVN, male OXY rats had higher MR-mRNA expression compared to
male CON rats. Although GR expression was not affected, the increase in MR expression may portray the importance of the balance between MR and GR and the interaction between GR and MR in HPA axis feedback control as has been described by others (Harris, Holmes et al. 2013, de Kloet 2014). The increased expression of MR in OXY males may have normalized the feedback process in the PVN during the restraint stress test leading to no enhanced response to RST in the male. This possibility is supported by a study by Harris et al indicating that the over-expression of MR partially compensates for the inadequate feedback control after stress, but not under basal conditions, in male mice that were GR deficient but over-expressed MR (Harris, Holmes et al. 2013).

Interestingly, in contrast to the male, MR-mRNA expression in the PVN of female OXY rat was not increased compared to female CON. This finding may indicate that the adaptation of the feedback mechanism that appears to occur in the male did not occur in the female and could lead to the enhanced response to RST in the female (Chapter 4). Moreover, MR expression in female OXY rats in the CA3 was decreased compared to CON, which is not consistent with results from the prenatal stress models that found reductions in GR, not MR expression in the CA3 area of male offspring of guinea-pigs and mice that were exposed to prenatal stress (Kapoor, Leen et al. 2008, Mueller and Bale 2008). However, the decrease in MR expression in the CA3 may reflect a down-regulatory adaptation to elevated fetal CORT. This is based on the fact that other selective kappa opiate agonists such as U50,488H can produce significant increases in plasma ACTH and CORT (Szeto 2003), therefore prenatal oxycodone exposure can potentially induce increases in fetal ACTH and CORT levels as well. The adaptation of MR expression to changes in fetal CORT levels has been previous studied; repeated antenatal treatment with Dexamethasone (DEX), which may decrease blood brain
barrier competency in the PVN (Frahm and Tobet 2015), significantly decreased plasma cortisol levels in fetal guinea-pigs and concomitantly increased fetal MR-mRNA expression in the CA3 with little effect on GR mRNA in limbic structures or the PVN (McCabe, Marash et al. 2001). So we may postulate that POE could increase fetal CORT levels, which would result in fetal adaptation by a decrease in MR-mRNA expression in the limbic system such as in the CA3 area.

The effects of prenatal opiate exposure on the feedback mechanisms have been studied previously by Slamberova and Rimanoczy et al. (Rimanoczy, Slamberova et al. 2003, Slamberova, Rimanoczy et al. 2004). In their studies, female rats prenatally exposed to morphine had a decreased ACTH response to RST and a decreased sensitivity to negative feedback as tested by DEX suppression (Slamberova, Rimanoczy et al. 2004). On the other hand, male rats prenatally exposed to morphine had a decreased ACTH response to RST but the sensitivity to the negative feedback action of glucocorticoids was not altered because DEX suppression was not changed (Rimanoczy, Slamberova et al. 2003). However, whether these findings in prenatal morphine models involved an increase in MR or GR expression in the PVN and limbic system was not studied.

Although we have not studied further the mechanisms by which the expressions of these genes were altered, we could speculate that epigenetic processes may play important roles. A wealth of knowledge from prenatal stress models has indicated that epigenetic mechanisms which include DNA methylation, histone modification and the actions of small non-coding RNAs can modify neurodevelopment of the HPA-axis during perinatal and postnatal periods (Maccari, Krugers et al. 2014, Bale 2015). These processes lead to an end product that determines how an organism interacts with and responds to exposures and experiences, whether they are nurturing or not. For example,
male mice prenatally exposed to stress had reduced gene methylation of the CRF promoter and increased gene methylation of the GR promoter which correlated with significantly increased CRF expression and decreased GR expression and behaviors (Mueller and Bale 2008). We propose to perform methylation analysis for these stress-related genes in a future study.

There are some limitations in this study that should be acknowledged. The number of rats in each group for immunocytochemistry was relatively small which could preclude us from finding small changes. We did not record estrous cycles of the female rat offspring and their brains were collected on random estrous cycle days. This could result in the variation of the expression of genes of interest including MR, CRH and ER because the expressions of these genes are influenced by the estrous cycle (Carey, Deterd et al. 1995, Nappi, Bonneau et al. 1997, Isgor, Cecchi et al. 2003). The rats were in resting condition, not formally stressed when their brains were collected for PCR therefore we may not find as much of a difference as expected in the expression of these stress-related genes. We did not study changes in GR or AVP expression in the pituitary; thus the mechanisms by which POE enhanced the pituitary ACTH response to CRH cannot be clearly identified if changes in feedback mechanisms occur at the pituitary level or involve changes in AVP expression. Finally, we had performed punched biopsy of the PVN to perform QRT-PCR. The PVN is such a tiny area that it is impossible to analyze gene expression from the subdivisions and the gene products from nearby regions may be partially included.

In summary, in this Chapter we explored the possible mechanisms by which perinatal oxycodone exposure affects the HPA-axis and circuitry control. Some findings may explain the enhanced response to restraint stress in the female rat exposed to oxycodone. These include an increase in the subpopulation of CRH neurons that also
contain immunoreactive ERbeta, which can exaggerate the positive stimulation, and a
decrease in MR-mRNA expression in the hippocampus which may impair the negative
feedback action on the HPA-axis. In contrast, the increase in MR-mRNA expression in
the PVN of male rats perinatally exposed to oxycodone may be part of the adaptation to
the intrauterine milieu that stabilizes the HPA-axis response to stress. It is interesting
that we found changes in MR-mRNA expression as brain mineralocorticoid receptors
have diverse functions in the stress system, not only mediating the initiation and
termination of the HPA-axis response to stress, but also regulating osmotic and
hemodynamic homeostasis, response to inflammation and injury and importantly,
cognition, learning and memory (Harris, Holmes et al. 2013, Gomez-Sanchez 2014).
This is one example indicating that the stress response requires orchestrated functions
of many systems and mediators to maintain homeostasis (Joels and Baram 2009). We
were interested to further explore whether POE had effects on another stress system,
the SAM-axis as exhibited by changes in the cardiovascular response to acute stress as
will be described in Chapter 6.
Table 5.1: The Sequence of Amplify Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH</td>
<td>GCTAACTTTTTCCGCGTGTT</td>
<td>GTTGCTGTGAGCTTGCTGAG</td>
</tr>
<tr>
<td>ERbeta</td>
<td>TTCCCGGCAGCACCAGTAACC</td>
<td>TCCCTCTTTGCCTTTGGGACTA</td>
</tr>
<tr>
<td>ERalpha</td>
<td>AATTCTGACAAATCGACGCCAG</td>
<td>GTGCTTCAACATTCTCCCTCCTC</td>
</tr>
<tr>
<td>GR</td>
<td>CACCCATGATCCTGTCACTG</td>
<td>AAAGCTCCCTCTGCTAACC</td>
</tr>
<tr>
<td>MR</td>
<td>GGTCACAGGTCCTCCACACT</td>
<td>GGAGGAGGACATGGAGTGA</td>
</tr>
<tr>
<td>Histone 3.1</td>
<td>GCAAGAGTGCGCCCTCTACTG</td>
<td>GCCCTCAGTTGCCTCGCAA</td>
</tr>
</tbody>
</table>
Figure 5.1: Photomicrographs showing CRH and or ERbeta positive
neurons in the PVN

5.1a: low power (10X)
5.1b: high power (100X)
5.1c: high power (100X, zoom in from Figure 5.1b) depicting 1) ERbeta/CRH neurons- black product in the nucleus and a brown chromogen product in the cytoplasm of the same cell (arrow), 2) ERbeta immunonegative/CRH neurons- brown chromogen product in the cytoplasm with an empty nucleus in the same cell (triangles), and 3) ERbeta positive neurons- black product in the nucleus (stars)
Figure 5.2: Total CRH positive neurons in the PVN (5.2 a), number of ERbeta immunopositive CRH neurons in the PVN (5.2b), percentage of ERbeta immunopositive CRH neurons in the PVN (5.2c) (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone (OXY, black bars) and control groups (CON, gray bars); Male OXY, n=5; Male CON, n=5; Female OXY, n=4; Female CON, n=5

* indicates mean differences between male OXY and male CON rats (p=0.018), ** indicates mean differences between female OXY and female CON rats (p<0.001)
Figure 5.3: Number (5.3a) and percentage (5.3b) of ERbeta immunonegative CRH neurons in the PVN (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY-H, black bars) and control group (CON, gray bars). Male OXY, n=5; Male CON, n=5; Female OXY, n=4; Female CON, n=5.

5.3a: * indicates mean differences between female OXY-H and female CON rats (p=0.018), ** indicates mean differences between male OXY and male CON rats (p<0.001)

5.3b: * indicates mean differences between male OXY and male CON rats (p=0.008), ** indicates mean differences between female OXY and female CON rats (p<0.001)
Figure 5.4: Number of ERbeta positive neurons in the PVN (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars)

Male OXY, n=5; Male CON, n=5; Female OXY, n=4; Female CON, n=5
Figure 5.5: CRH (5.5a), ERbeta (5.5b) and ERalpha (5.5c) mRNA expression (fold change over control) in the PVN (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars)

5.5a: Male OXY, n=7; Male CON, n=8; Female OXY, n=8; Female CON, n=6
5.5b: Male OXY, n=7; Male CON, n=8; Female OXY, n=8; Female CON, n=6
5.5c: Male OXY, n=7; Male CON, n=8; Female OXY, n=7; Female CON, n=6
Figure 5.6: GR (5.6a) and MR (5.6b) mRNA expression (fold change over control) in the PVN (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars).

5.6a: Male OXY, n=7; Male CON, n=8; Female OXY, n=8; Female CON, n=6

5.6b: Male OXY, n=7; Male CON, n=8; Female OXY, n=7; Female CON, n=6

* indicates mean differences between male OXY and male CON rats (p=0.022)
Figure 5.7: CRH, ERbeta, ERalpha, GR and MR mRNA expression (fold change over control) in the hippocampal CA1 area in the male rats (5.7a) and female rats (5.7b) (mean ± SEM) in oxycodone group (OXY, black bars) and control group (CON, gray bars)

Male OXY, n=9; Male CON, n=4
Female OXY, n=9; female CON, n=6
Figure 5.8: CRH (5.8a), ERbeta (5.8b) and ERalphaERalpha (5.8c) mRNA expression (fold change over control) in the hippocampal CA3 area (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars) Male OXY, n=8; Male CON, n=5; Female OXY, n=11; Female CON, n=6
Figure 5.9: GR (5.9a) and MR (5.9b) mRNA expression (fold change over control) in the hippocampal CA3 area (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars)

Male OXY, n=8; Male CON, n=5; Female OXY, n=11; Female CON, n=6

* indicates mean differences in female OXY and female CON (p=0.017)
Figure 5.10: CRH (5.10a), ERbeta (5.10b) and ERalpha (5.10c) mRNA expression (fold change over control) in the central nucleus of the amygdala (CEA) (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars).

Male OXY, n=5; Male CON, n=6; Female OXY, n=10; Female CON, n=4; * indicate mean differences between female OXY and female CON (p= 0.003)
Figure 5.11: GR (5.11a) and MR (5.11b) mRNA expression (fold change over control) in the central nucleus of amygdala (CEA) (mean ± SEM) in male (2 bars on the left) and female (2 bars on the right) rats in oxycodone group (OXY, black bars) and control group (CON, gray bars) Male OXY, n=5; Male CON, n=6; Female OXY, n=10; Female CON, n=4
Figure 5.12: CRH, ERbeta, ERalpha, GR and MR mRNA expression (fold change over control) in the prefrontal cortex (PFC) in male (5.12a) and female rats (5.12b) (mean ± SEM) in oxycodone group (OXY, black bars) and control group (CON, gray bars); Male OXY, n=9; Male CON, n=8; Female OXY, n=11; Female CON, n=10
Figure 5.13: Schematic summary of changes in the HPA-circuitry after perinatal oxycodone exposure

PVN: Paraventricular Nucleus, PFC: Prefrontal Cortex, CEA: Central Nucleus of Amygdala; CRH: Corticotropin Releasing Hormone, ERbeta: Estrogen Receptor Beta, MR: Mineralocorticoid Receptor; F-OXY and M-OXY: female and male oxycodone exposed rats

- **PFC**: no changes
- **Hippocampus**: CA1: no changes, CA3: MR in F-OXY

- **PVN**: Both Sexes: CRH/ERbeta positive neurons

- **Male**: MR-mRNA in M-OXY

- **CEA**: CRH-mRNA in F-OXY
6.1 Introduction

This chapter is an excerpt from previously published work by Sithisarn et al in collaboration with Dr. David Randall (Sithisarn, Bada et al. 2013). The telemetry surgery and conditioning paradigms were performed in Dr. David Randall’s laboratory.

Besides the HPA axis, another important system that is responsible for reinstatement of homeostasis is the sympathetic-adrenal-medullary (SAM)-axis. SAM-axis activation in response to stress had been classically studied and now it is known that complex reactions also occur in the SAM-axis during both physiological and psychological stress (Huang, Webb et al. 2013). Thus changes and adaptation to a stressful intrauterine environment likely do not occur in only the HPA-axis circuitry but could also occur in other systems that are closely involved in the stress response like the SAM-axis. The SAM-axis integrates the control of cardiovascular function and blood pressure (Wallin and Charkoudian 2007) and has been shown to be a key player in various diseases including hypertension and heart failure (Charkoudian and Rabbitts 2009).

A number of studies have reported the suppressive effects of exposure to prenatal morphine on the stress axis and behavior. For example, prenatal morphine exposure is associated with adrenal atrophy and adrenal hypoactivity in neonatal rats (Lesage, Bernet et al. 1996), decreased elevation of adrenocorticotropin (ACTH) and corticosterone (CORT), depression-like behavior during forced swim tests in adult male rats (Klausz, Pinter et al. 2011), and suppressed response of ACTH to a restraint stress
test (RST) in adult male and female rats (Slamberova, Rimanoczy et al. 2004). However, data remain limited regarding the effects of perinatal oxycodone exposure on the stress axis. We have previously reported that perinatal oxycodone increases the pituitary (ACTH) response to a pharmacological challenge, corticotropin releasing hormone, only in late adolescent male, but not female rat offspring (Sithisarn, Bada et al. 2008). Moreover, we found that perinatal oxycodone exposure was associated with an enhanced corticosterone response to restraint stress test in adult female offspring, as described in Chapter 4. Not only the HPA-axis, but also the sympathetic-adrenal-medullary- (SAM) axis intimately regulates the stress response (Carrasco and Van de Kar 2003, de Kloet, Joels et al. 2005). Blood pressure (BP) is a major cardiovascular output of the SAM-axis that has never been adequately studied in the context of perinatal drug exposure; however, other perinatal influences such as poor nutrition, maternal smoking or perinatal stress that result in intrauterine growth restriction and/or low birthweight, are associated with hypertension later in life (Bakker and Jaddoe 2011, Alexander, Dasinger et al. 2015). Studies have shown that alteration in sympathetic nervous system control is one of the key factors implicated in this developmental programming of changes in blood pressure (RG, Stehouwer et al. 2003, Boguszewski, Johannsson et al. 2004, Mizuno, Siddique et al. 2013). Thus, we proposed to study the effects of POE on the SAM by investigating the cardiovascular response to acute behavioral stress in the male offspring using a classical conditioning paradigm that has been previously described (Randall, Brown et al. 1994). We tested the hypothesis that perinatal oxycodone exposure enhances the blood pressure response to classical aversive conditioning, and also impairs the offspring’s ability to differentiate between stressful vs. non-stressful stimuli.
6.2 Material and Methods

6.2.1 Experimental Design: Animals and Prenatal Treatments.

The rats were treated as described in Chapter 4.

The pregnant rats were randomly assigned to either control (CON, normal saline vehicle) or oxycodone (OXY) treatment groups. From GD 8 to 21, experimental dams were slowly injected over 10 minutes via the atrial cannula with oxycodone hydrochloride (2 mg/kg/day; n= 5 dams) (Mallinckrodt, St. Louis, MO) in normal saline solution (NSS). This dose was selected based on a previous study (Chapter 4) that indicated that the dams were able to tolerate this dose without disturbance of litter size or birth weights of rat pups and that it is adequate to create opiate effects. Control dams were given 1 ml/kg NSS once daily (n=6 dams).

Rat pups were treated as described in Chapter 4. After weaning, the pups were randomly assigned to the experimental groups. For statistical analysis, data from all pups within a given litter were averaged to generate one data set per dam as described below.

6.2.2 Experiment: Classical Conditioning

Subjects: Male rat pups were randomly selected on PD 27-30 from CON (n=12 pups from 6 dams) and from OXY-treated litters (n=11 pups from 5 dams) for the classical conditioning study. Only male pups were included in this experiment due to the limited availability of the telemetry probes and the predominance of cardiovascular disease in the males (Barrett-Connor 1997, Moller-Leimkuhler 2007).

Implantation of the Telemetry: Arterial blood pressure telemetry probes (PhysioTel™, Model PA-C40, Data Science International, MN) were implanted in each experimental pup at PD 27-30 days of age using standard rodent survival surgery techniques. The animals were anesthetized (sodium pentobarbital, 50 mg/kg) and the
abdominal aorta exposed via a laparotomy. The sensory element of the implantable telemetry probe was placed into the aorta via puncture such that its tip pointed toward the heart (i.e., “upstream”). The body of the probe (i.e., that contains the necessary circuitry, transmitter and battery) was secured to the interior abdominal wall. The incision was closed and the skin approximated by wound clips. The animals were placed on a warm pad and were monitored until they recovered from surgery. Upon arousing they were returned to their home cage. The rats were allowed a minimum of 3 days to recover before experiments commenced.

Behavioral conditioning: Details of the conditioning paradigm have been published (Randall, Brown et al. 1993, Randall, Brown et al. 1994). Briefly, the animals were habituated to handling and restraint in a comfortable conical cloth sock for 1-2 h daily for two days. The animal was free to emerge from the restraint, but was immediately reintroduced to the sock until, by the end of the second day, it tended to ‘snuggle’ at the apex of the cone with only occasional attempts to exit. Each rat was then exposed to five sets of a tone that would eventually become the "stress stimulus" and a tone that eventually would become the "non-stress stimulus". The stressful stimulus (CS+) consisted of a 15-second (s) pulsed tone; on the last tone of this first day of training, and on all subsequent presentations, CS+ was followed by a 0.5-s tail shock, the unconditional stimulus (US). The intensity of the shock was adjusted to the lowest level that caused the rat to flinch and vocalize (squeak); the intensity usually ranged between 0.2-0.3 mA and never exceed 0.3 mA. The 15 sec, non-stressful stimulus tone (CS-) was identical to the CS+ tone except it sounded continuously (i.e., the tone was not pulsed), and was never followed by a shock. Tones were presented in random pairs (e.g. CS+,CS-;CS-,CS+…). A minimum of 5 minutes elapsed between tone presentations. Training in the conditioning paradigm continued for two additional days during which 5 CS+ and 5 CS- were presented daily.
6.2.3 Data Acquisition and Analysis

Conditioning trials were conducted starting at PD40 and, in some pups, again starting at PD75. In each case the rat was restrained in the cloth shock and an initial single day’s set of 5 CS+ and 5 CS- trials was conducted to ‘refresh’ the conditional response; the BP and HR data from these trials were not used in data analysis. Over the next two days additional sets of 5 CS+ and 5 CS- trials were conducted during restraint and these data were retained for subsequent analysis of the conditional cardiovascular response. Digital data sampling began 15 seconds before the onset of the tone and continued for 30 seconds (i.e., until 15 sec. after tone-off). Data from conditioning trials from a given rat were ensemble averaged (see below) for that pup; data from pups born of a common dam were, in turn, averaged together to yield a single data set for each OXY and each CON-treated dam. Blood pressure was digitally sampled at 500 Hz using an analog-to-digital converter (Data Translation 2810) and a microprocessor. HR was determined from the pulsatile BP signals. The programs (Vii soft, Lexington, KY) were developed for a 32 bit operating system (Windows NT) using Microsoft Visual C++ with foundation class in order to utilize large data files. The digital files of the BP recorded during 10 CS+ were ensemble averaged for each rat to yield a “high resolution” analysis of the conditional response for that individual (Randall, Brown et al. 1993, Randall, Brown et al. 1994); likewise for CS- trials.

The data analysis program quantified the conditional response from the ensemble data files. For each individual rat the mBP and HR averaged over the 15 seconds immediately preceding the tone was taken as the baseline, and all aspects of the response pattern were assessed as changes relative to this baseline. The initial increase in mBP was assessed as the maximum change observed within the first 2 s after the tone onset (i.e., C1-Max). The time when C1-Max occurs (i.e., t C1pk) was
determined with respect to tone onset. C2-Avg was the average value of mBP during the final 10 s of the tone; this interval is indicated in Figure 6.1. The unconditional response, or UR, is given as the maximum BP response occurring within the 3.5 s following the end of the tone. The HR corresponding in time to each of the BP values, above, was also recorded. Note that the BP data between the third and fifth sec. of the tone were discarded since they included the fall in pressure that separates C1 from C2 (Randall, Brown et al. 1993, Randall, Brown et al. 1994).

**6.2.4 Statistical Analyses**

The data were analyzed using a linear mixed model in which the presence or absence of oxycodone exposure is the independent variable and the physiologic parameters (HR, mBP) are dependent variables. All findings are reported as mean ± SEM. Statistical significance was defined as p<0.05.

**6.3 Results**

**6.3.1 Parturition, Litter Size and Body Weights**

There were no differences between the OXY and CON pups in timing of parturition, litter size and body weight, either male or female, from birth to PD 32 (p>0.05). For the OXY and CON male pups, mean birth weights (SEM) were 5.41(0.31) g and 4.92 (0.11) g respectively. Neither were there differences in body weights of the pregnant rats for this experiment between the two groups before or after delivery.
6.3.2 Baseline Mean Arterial Blood Pressure and Heart Rate, PD 40

Average baseline (i.e., pre-tone) mBP was lower in perinatally oxycodone exposed offspring (OXY: 114.8 ± 1.0) compared to pups from dams exposed to CON (118.3 ± 1.0 mmHg) (p=0.02). Baseline heart rates were not different between the two treatment groups (CON 462 ± 10.8 bpm; OXY: 456 ± 11.3 bpm).

6.3.3 Conditional Cardiovascular Response, PD 40

**Group Averaged CS+ and CS- trials:** Figure 6.1 shows the high resolution analyses of the change (Δ, relative to baseline) in mBP (top panel) and in HR (bottom panel) averaged across all pups from NSS treated dams (n=6) in response to the CS+ tone (blue) and the CS- tone (black). Data are shown starting 15 sec. prior to the tone onset and extending for 15 sec. after the half-second shock delivery (or, for CS-, tone-off). The mBP increased to an initial peak (C1-max) immediately following the tone onset for both CS+ and CS-. Recall that the CS- tone was identical in frequency and amplitude to the CS+ tone so several tenths of a second elapsed before the animal could determine if a given tone was pulsed (CS+) or steady (CS-); hence the initial response to CS-. The increase in mBP was sustained in response to CS+ as seen by the clear C2 that extended throughout the latter seconds of the trial. Conversely, mBP decreased to baseline during CS- after the initial C1 increase. HR modestly decreased within seconds in response to the onset of both tones; it remained below baseline throughout CS+ but returned towards baseline for CS-. The unconditional response (UR) to the tail shock for CS+ trials consisted of an increase in mBP and in HR. There were no corresponding sustained changes following the CS- tone.

Figure 6.2 shows the actual value (i.e., not normalized to baseline) for mBP and HR for conditioning trials from pups born from 6 CON and 5 OXY dams. The lower
baseline mBP in pups from OXY dams, which was described above, is easily discerned. Likewise, the similarity in baseline HRs between the two groups is clear. The individual components of the mBP and HR responses to CS+ and CS- (not shown, Figure 2) are presented below.

**Mean arterial BP conditional response, PD 40:** As can be discerned qualitatively in Figure 6.2, the initial, short-latency C1 peak increases in mBP after CS+ onset were not different between CON (+5.1 ± 0.4 mm Hg) and OXY pups (+5.7 ± 0.4 mm Hg). Although the magnitude of the peak change in C1 mBP (ΔC1pkBP) did not differ between CS+ and CS- tones for either group, the average value of mBP throughout the C1 event was significantly larger during CS+ as compared to CS- tones for both groups with no significant group x tone interaction. Finally, the time at which the peak C1BP (tC1pk) was attained relative to tone onset (i.e., evaluated for both CS+ and CS-) was similar for CON (0.74 ± 0.13 sec) and OXY pups (0.84 ± 0.14 sec).

The second component (C2) of the mBP response, that is sustained throughout the last 10 seconds of the tone, and the corresponding change in HR (see below) are of particular interest with respect to an animal’s ability to acquire the conditional response and to discriminate between the two conditions (Randall, Brown et al. 1993, Randall, Brown et al. 1994, El-Wazir, Li et al. 2005). CS+ produced a larger C2 pressor response (ΔC2BP) in rats from OXY-treated dams (+3.9 ± 0.4 mm Hg) as compared to CON pups (+1.7 ± 0.4 mm Hg) (Figure 3, top). This difference persisted even when corrected statistically for differences in baseline values. Both OXY and CON rats discriminated between CS+ and CS-, as reflected in a significant difference in ΔC2BP between CS+ and CS- (CON CS-: -0.6 ± 0.4 mm Hg; OXY CS-: +0.4 ± 0.4 mm Hg). The group x tone interaction, however, was not significant.
There were no between group differences in any aspect of the animals’ mBP response to shock delivery itself (UR BP). Likewise, there were no differences in the mBP during the 15 seconds following shock delivery (i.e., recovery).

**HR conditional response, PD 40:** The cardio-deceleration that occurs during CS+ concomitantly with the C2 pressor response, but which is not sustained during CS-, as shown in Figure 1, is another hallmark of the discrimination between CS+ and CS-. Figure 2 suggests that the slowing during CS+ is less in the OXY as compared to the CON rats. In fact, statistical analysis of actual HR values, of actual HR controlled for baseline differences, and of changes in HR during C2 (Δ C2HR) confirms that the OXY rats’ bradycardia during C2 was smaller than in CON (Figure 3, bottom). In particular, the -24.8 ± 19 bpm slowing observed in the CON during the last 10 sec. of CS+ significantly exceeded the -16.6 ± 2.0 bpm observed in OXY; moreover, there was a significant group x trial interaction (F1,21 = 9.37). This difference in the change in HR persists when the effect of the somewhat different baseline HR is controlled for statistically.

**6.3.4 Baseline Mean Arterial Blood Pressure and Heart Rate, PD 75**

We maintained a subset of pups from CON and OXY through an age of 75 days post-delivery to determine if any between group differences were accentuated or diminished with age. Mean baseline BPs in adults were higher than those on PD 40, but the overall baseline BPs in OXY, were not significantly different from CON (OXY: 143.4 ±1.7 vs CON: 149.1 ± 2.8 mmHg; p = 0.1). Baseline HRs were remarkably lower at PD 75 than at PD 40 for both CON (396 ± 21 bpm) and for OXY (395 ± 13 bpm), but, again, there were no between group differences.
6.3.5 Conditional Cardiovascular Response, PD 75

**Mean arterial BP conditional response, PD 75:** After adjustment for the baseline BP, C_{1pk} BP, Δ C_{1pk} BP, C_{1avg} BP and Δ C_{1BP} were not different between OXY and CON rats, either during CS+ or CS-. Time to the peak C_{1} mBP increase was not different between OXY and CON either during CS+ or CS-.

The significant difference in the amplitude of the Δ C_{2}BP response during CS+ observed at PD40 disappeared by PD 75 (CON: +3.4 ± 2.3 mm Hg; OXY: +4.5 ± 1.4 mm Hg).

Importantly, CON rats were able to differentiate between CS+ and CS-, as demonstrated by an increased C_{2}BP during CS+ but not for CS- (CS+: 155.4 ± 2.7 vs CS-: 147.8 ± 2.7 mmHg; p = 0.02). Conversely, even though OXY rats could differentiate between CS+ and CS- at younger age, they did not retain this ability during adulthood (CS+: 147.1 ± 1.6 vs CS-: 145.9 ± 1.6 mmHg, p = 0.49). These discrepancies persisted after controlling for the baseline values or when comparing using Δ C_{2pk} BPs (CON CS+ vs CS-: p = 0.029, OXY CS+ vs CS-: p = 0.14).

**HR conditional response, PD 75:** A major difference in the conditional HR response at PD 40 was that the CON animals slowed heart rate more during C_{2} than did the OXY animals. At PD 75 there were no between group differences in Δ C_{2}HR during CS+, and, in fact, the conditional bradycardia at PD 40 was no longer elicited during C_{2} at PD 75 (CON: -2 ± 17 bpm; OXY +2 ± 10 bpm)

UR HRs were similar between CON and OXY rats, during both CS+ and CS-. UR HRs were also similar between CS+ and CS- in each treatment group. Finally, there were no between group differences in recovery mBP or HR.
6.4 Discussion

6.4.1 PD 40:

This study has demonstrated quantitative differences in baseline mBP and in select aspects of the cardiovascular response to an acute behavioral stress in rats perinatally exposed to oxycodone, as opposed to control pups born of dams exposed to saline. The conditional response is advantageous for a study such as this because a great deal is known about the underlying mediation of the changes in mBP and in HR, and because the response pattern is reproducible and stable over time. Moreover, the response pattern can be elicited multiple times at the investigator’s discretion. Major findings are that at forty days of age, rat offspring in the OXY group as compared to the CON had a modestly, but significantly, lower baseline mBP (with no difference in baseline HR), and a larger increase in mBP during the C₂ component of the conditional response with a concomitantly smaller decrease in HR. There was no between-group difference in the C₁ component of the BP conditional response. These findings can be interpreted in terms of what is known about the mediation and control of the conditional cardiovascular response pattern in the mature SD rat.

The short-latency conditional increase in mBP, which we call C₁, is preceded by a large-amplitude, but short-lived, ‘sudden burst’ (SB) in sympathetic nerve activity (SNA) in Sprague-Dawley (SD) rats (Randall, Brown et al. 1994); the amplitude of the SB correlates with the amplitude of the C₁ pressor response (Burgess, Hundley et al. 1997). The C₁ BP increase is produced by an increase in peripheral resistance; in fact, there is little or no concomitant change in either stroke volume or HR and, thereby, none in cardiac output (Li, Randall et al. 1998). As noted previously, C₁ originates as an orienting or startle response (though it subsequently attains properties of a conditional response); that is, no ‘learning’ is required for the animal to demonstrate this component.
of the response (El-Wazir, Li et al. 2005). It is noteworthy, therefore, that there were no between-group differences in the present study in any aspect of C₁, including its latency with respect to tone onset. That is, oxycodone exposure in utero did not affect this ‘intrinsic’ aspect of an acute stress response.

The C₂ pressor event, which occurs following the sudden burst in SNA, is accompanied in time by a moderate (ca. +24%), but sustained increase in sympathetic activity (Randall, Brown et al. 1994). Relative to baseline, cardiac output increases during C₂ by 2 ± 1 ml/min, while peripheral resistance decreases on the average by 4 ± 2 dyn · s/cm⁵ in the SD strain (Li, Randall et al. 1998). The sustained C₂ mBP increase is dependent, therefore, upon the heart’s developing and maintaining an increase in cardiac output over baseline. In contrast to C₁, C₂ is acquired as the animal learns the association between the CS+ tone and the US shock (El-Wazir, Li et al. 2005)—the rat must learn the tone/shock pairing to display a C₂. It is again particularly noteworthy, therefore, that the C₂ mBP increase was significantly larger in the OXY animals than their controls. This implies that the drug exposure in utero impacted ‘higher’ cognitive function with effects that can be detected in the offspring’s learned response pattern.

The nature of the C₂ HR change during CS+, if any, is species-dependent (compare Randall, Brown et al. 1994; Li, Randall et al. 1998; Li, Lawler et al. 1997; Brown, Li et al. 1999). To date we had studied only adult rats, and HR is essentially unchanged (Randall, Brown et al. 1994) or decreases by only ~5 bpm (Li, Randall et al. 1998) relative to baseline in adult SD rats during the last 10 sec. of CS+. The CS+ C₂ bradycardia is eliminated by atropine in Zucker lean and obese rats, but only modestly (though significantly) attenuated by beta-adrenergic blockade (El-Wazir, Li et al. 2008) . The HR slowing is therefore attributable primarily to elevated parasympathetic nervous drive to the SA-node, probably via the baroreflex secondary to any C₂ mBP increase. In
the context of these previous studies, two current observations are remarkable. First, in the young SD rats of both groups, in contrast to the SD adult, HR significantly decreased during C2 relative to baseline (Figure 6.1). Second, the C2 HR decrease was significantly smaller in the OXY vs. CON group, despite the larger C2 mBP increase in the OXY vs. CON. This latter observation implies either that the parasympathetic control of HR is somewhat impaired in the OXY animals, or that the ‘gain’ of their baroreflex is smaller than the controls, or perhaps both conditions obtain.

A clear difference in the nature of the cardiovascular response to CS- vs. CS+ is indicative of the subject’s ability to discriminate between the two behavioral situations. In the conditioning paradigm, discrimination such as this demonstrates that the response pattern is truly a learned behavior, and not simply an erratic response to any given event (Randall, Brown et al. 1993). The ability of the SD rat to demonstrate such discrimination is acquired over successive trials during the ‘acquisition’ phase of training—as the animal learns, or acquires the conditional response (El-Wazir, Li et al. 2005). Each group clearly demonstrated the ability to discriminate CS+ from CS-, both by the relatively smaller C2 mBP increase and smaller HR decrease during CS-. In other words, perinatal exposure to oxycodone did not demonstrably impair this aspect of the OXY animals’ ability to learn the behavioral paradigm at PD 40.

6.4.2 PD 75:

As animals in both groups matured, baseline mBP rose and baseline HR fell; the significant difference observed at PD 40 in baseline mBP disappeared. Moreover, the significant difference in Δ C2BP at PD 40 also disappeared. These findings indicate that, as the OXY rats matured, the effects of their perinatal exposure to oxycodone on their response to the acute stress dissipated. Finally, the significant HR slowing during C2,
which is not characteristic of the (adult) Sprague-Dawley, was no longer evoked during CS+ at PD 75. This indicates that the nature of the conditional HR response changes with maturation.

The baseline mBPs in both groups at PD 75 (i.e., CON 149 mm Hg, OXY 143 mm Hg) were higher than we expected. That stated, the 75 day-old animals are younger than animals in which we have typically recorded pressure, so it may be that at this earlier developmental stage the mBP is higher than we observe in the mature rat. In fact, Litchfield reported a progressive increase in mBP from birth to PD 35 (mBP = 109.6) in anesthetized rat pups, but he did not follow their pressures further, and the trajectory in the rise of mBP appeared to be leveling by PD 35 (Litchfield 1958). Kasparov and Paton (1997) also reported an upward progression in anesthetized rat pups from PD 6 to 45 (mBP = 74.6 mm Hg), but with no additional statistically significant increase at PD 45 (Kasparov and Paton 1997). We reported beat-by-beat mBP via telemetry averaged over 24 hours in rats ~60-90 days of age while in their home cages to be ~98 mm Hg, and that mBP gradually declined thereafter as the animals matured (Anigbogu, Speakman et al. 2012). By comparison, Hoy et al reported (Hoyt, Speakman et al. 2013) a mBP of 127.6 ± 13.5 (SD) mm Hg via catheter in behaviorally conditioned adult rats during the 15 sec. baseline (i.e., as in the present study), which is clearly higher than our value from the 24 hour telemetry. The present pups were not subject to the sock restraint or periodic handling between measurements at PD 40 and at PD 75, so the unexpectedly high mBP perhaps is attributable to the relatively unaccustomed restraint on PD 75.
6.4.3 Prenatal Opiates Effects on the Autonomic Nervous System (ANS):

To date there are no human or animal studies that directly explore the effects of prenatal oxycodone on blood pressure and autonomic system controls; however, there is evidence both from human and animal studies suggesting that the autonomic nervous system is affected by the exposure to opiates in utero. Many human neonates prenatally-exposed to opiates experience symptoms of the neonatal abstinence syndrome, which are autonomic regulated functions (e.g. increased sweating, nasal stuffiness, fever, mottling, and temperature instability) (1998, Bandstra, Morrow et al. 2010). To study autonomic control in children, vagal tone adaptation, among other methods, has been used as an indicator of autonomic regulation in the setting of prenatal cocaine exposure (Sheinkopf, Lagasse et al. 2007). The variability in heart rate that occurs at the frequency of breathing, or respiratory sinus arrhythmia (RSA), reflects the parasympathetic influence on HR variability (HRV) via the vagus nerve (Randall, Brown et al. 1991, Berntson, Cacioppo et al. 1993, Calkins and Keane 2004, Yasuma and Hayano 2004). Suppression of RSA on electrocardiography has been considered an adaptive response indicative of removal of the vagal brake to increase metabolic output in order to engage more effectively with the environment (Porges 1995, Porges 2007). In general, higher levels of baseline parasympathetic activity as measured by RSA and/or the ability to suppress parasympathetic activity are related to enhanced autonomic emotional regulation and its developmental outcomes (Calkins and Keane 2004, Stifter, Dollar et al. 2011). During an attention demanding task performed by school-aged boys prenatally exposed to opiates, such as heroine or methadone, RSA suppression was impaired, suggesting possible long term effects of opiates on the (dis)organization of the vagal system (Hickey, Suess et al. 1995). However, this finding was inconsistent with a subsequent study which showed that when an extrinsic incentive, in addition to
interesting tasks, were offered, RSA suppression in opiate-exposed school-age boys was comparable to the controls (Suess, Newlin et al. 1997). Besides RSA, HRV measures from beat to beat fluctuations have been used to assess the status of sympathetic and parasympathetic balance (Berntson, Bigger et al. 1997, Lappi, Valkonen-Korhonen et al. 2007). Our group recently studied HRV from the electrocardiographic data during sucking (both nutritive and non-nutritive) in neonates exposed to opiates in utero compared to the control term infants in order to evaluate ANS functions. Opiate exposed infants demonstrated greater HRV or greater mean SDRR (standard deviation of consecutive RR intervals) and SDDRR (standard deviation of the differences of consecutive RR intervals) during non-nutritive period as well as greater mean SDDRR during nutritive sucking. Higher powers in the low and high frequency bands during nutritive feedings were also exhibited in prenatal opiate exposed infants. These findings suggested that prenatal opiate exposure may be associated with alterations in both sympathetic and parasympathetic control of the ANS (Hambleton, Reynolds et al. 2013).

Animal studies have shown that prenatal opiates induced changes in sympathoadrenal activity, although the direct effects of these changes on blood pressure and heart rate have not been previously examined. For example, under resting conditions, adult male rats prenatally exposed to morphine had decreased adrenal noradrenaline (NA) and adrenaline contents, but increased circulating levels of adrenaline (Dutriez-Casteloot, Bernet et al. 1999). Under ether inhalation stress, these rats had hypo-responsive SAM activity; adrenal norepinephrine decreased at 90 minutes after inhalation and the compensatory biosynthesis of adrenal catecholamines did not adapt appropriately to stress when compared to controls (Laborie, Dutriez-Casteloot et al. 2005).
The possible underlying mechanisms of changes in autonomic control after prenatal exposure to oxycodone remain to be investigated. The enhanced C2 mBP increase in the OXY animals implies either that they have a larger increase in SNA evoked by the acute stress or that the effector response (i.e., vascular smooth muscle and/or myocardium) to a given increase in SNA was enhanced in OXY animals. Changes in the regulatory functions of kappa opioid receptors on the myocardium may also contribute to the enhanced mBP increase. Oxycodone acts, along with Mu opioid receptors, on kappa (κ)-opioid receptors (KOR) (Ross and Smith 1997). The K-opioid system works closely with the sympathetic nervous system in the regulatory functions of the heart (Wong and Shan 2001). Endogenous kappa-opioid peptides (dynorphins) are found in the sympathetic nerve fibers and ganglion cells (Steele, Aromataris et al. 1996). Chemical sympathectomy reduces the amount of dynorphin in the heart, indicating that κ-opioid peptides may co-exist with the catecholamines in the sympathetic nerve terminals (Wegener and Kummer 1994, Pepe, van den Brink et al. 2004). The activation of KOR with a selective exogenous agonist U50,488H inhibits the effects of β-adrenergic receptor (β-AR) agonist to increase rat myocyte contractility (Yu, Li et al. 1998). A selective KOR antagonist prevented these inhibitory effects, indicating that the effects are KOR mediated (Yu, Li et al. 1998). An interaction between KOR and β-AR (Pepe, van den Brink et al. 2004), disturbed by a significant reduction in or absence of the inhibition of β-AR stimulation by KOR stimulation, may lead to an excessive increase in cardiac activity leading to disproportionately increased blood pressure (Wong and Shan 2001). Chronic exposure to other opioid agonists, such as morphine, causes receptor internalization and changes in receptor binding or post-translational modification and receptor biosynthesis (Patel, Patel et al. 2002, Przewlocki 2004, Nagi and Pineyro 2011). Thus, we can speculate that long-term in-utero exposure to a KOR agonist such as oxycodone may down-regulate the expression of KOR in cardiac myocytes. In turn,
this down-regulation may reduce the inhibition of β-AR stimulation during stress and lead to significantly increased C₂ mBP in the OXY animals.

6.4.4 The Effects of Prenatal Opiates Exposure on Learning/Memory and Cognition:

There have been some human studies that identify the effects of prenatal opiate exposure on cognitive development and learning to date; most of those were conducted in children born to heroin or methadone dependent mothers who also used other illicit drugs. Thus, the effects of other drugs and psychosocial factors confounded the outcomes. Hyperactivity, lack of concentration, and aggression were reported in these children (Olofsson, Buckley et al. 1983). Cognitive deficits in opiate-exposed children were noted at various ages in a few studies (van Baar and de Graaff 1994, Pulsifer, Radonovich et al. 2004, Steinhausen, Blattmann et al. 2007). Previous reports indicate that prenatal exposure to opiates is associated with impaired learning/memory (Niu, Cao et al. 2009, Wang and Han 2009, He, Bao et al. 2010). More recently, Davis et al (Davis, Franklin et al. 2010) used an animal model to study the effect of prenatal oral oxycodone exposure on learning and/or memory in adult male rats. OXY rats showed a decreased use of spatial strategies and increase in non-spatial strategies in the Morris water maze. Interestingly, OXY rats had a modest but significant retention deficit in T-maze tasks when assessed 5 days after acquisition training ended (Davis, Franklin et al. 2010). This is consistent with our findings that the OXY rats had memory retention deficit. Although OXY rats were able to learn to differentiate between CS+ and CS- at age PD 40, they were not able to retain this ability when tested at PD 75 even if the procedure was repeated prior to the test with a set of 5 CS+ and 5 CS- trials in order to ‘refresh’ the conditional response at this age. These findings suggest that perinatal oxycodone exposure may be associated with impairment of the formation and/or storage of memory.
We further explored the effects of perinatal oxycodone exposure on learning and memory using a different testing paradigm as described in Chapter 7. The mechanisms for this memory deficit remain to be elucidated but there is evidence that prenatal exposure to other opiates is associated with several alterations in hippocampal cholinergic function (Vatury, Barg et al. 2004), glutamatergic neurotransmission (Tao, Yeh et al. 2001, Yang, Huang et al. 2003), hippocampal synaptic complex (Lin, Tao et al. 2009), and increased hippocampal neuronal apoptosis (Wang and Han 2009), all of which may lead to memory/cognitive deficits.

Because the fetal programming of the cardiovascular system after perinatal insults is likely sex-dependent (Jones, Jurgens et al. 2012, Alexander, Dasinger et al. 2015), that we did not include female offspring in this experiment could be a limitation. The previous study by Ojeda et al reveals that estrogen contributes to the normalization of blood pressure in adult female rats born intrauterine growth restricted (Ojeda, Johnson et al. 2007). We propose to include female offspring in our experiment in the future.

6.5. Conclusion

In conclusion, perinatal oxycodone exposure is associated with an increased blood pressure response to the ‘learned’ component of an acute behavioral stress in adolescent male rats, suggesting increased SNS input or increased response of the effectors. This difference dissipated when the stress was repeated as the rats matured to adult age. Adult prenatally oxycodone-exposed rats also had impaired retention of the learning of this conditioning at a younger age, which may result from a memory deficit associated with prenatal opiate exposure. We therefore further explored in Chapter 7, whether perinatal oxycodone exposure was associated with changes in other aspects of
neurobehavior, including a response to separation from the dams during the neonatal period, exploration and hyperactive behavior, and learning and memory in the offspring.
Figure 6.1: The high resolution analyses of the change ($\Delta$, relative to baseline) in mean arterial blood pressure (mBP; top panel) and in heart rate (HR, bottom panel) for offspring on postnatal day 40 from control dams (n=6) to CS+ tone (blue) and CS- tone (black).

The telemetry surgery and conditioning paradigms were performed in Dr. David Randall’s laboratory.
Figure 6.2: The actual value (i.e., not normalized to baseline) for mBP and HR for conditioning trials from offspring from 6 control (Saline) and 5 oxycodone (Oxy) dams on postnatal day 40

The telemetry surgery and conditioning paradigms were performed in Dr. David Randall's laboratory.
**Figure 6.3:** The corresponding changes from baseline in BP (top panel) and HR (bottom panel) during the conditional response between the two tones CS+ (dark bars) or CS- (gray bars) in control and oxycodone rats on postnatal day 40 (*p < 0.05*)

The telemetry surgery and conditioning paradigms were performed in Dr. David Randall’s laboratory.
CHAPTER SEVEN
THE EFFECTS OF PERINATAL OXYCODONE EXPOSURE ON BEHAVIORS

7.1 Introduction

We found, as described in Chapters 4 and 5, that the effect of perinatal oxycodone exposure (POE) on the HPA-axis response to stress could partially be explained by changes in the feedback mechanisms and feed-forward control. POE was also associated with alterations in the cardiovascular response to stress and may impair discrimination ability/memory, as described in Chapter 6. These changes in the HPA-axis may also be associated with short and long term behavioral problems since the HPA-axis has important roles in programming neurobehavioral development (Mesquita, Wegerich et al. 2009). Dysregulation in the HPA-axis functions has been linked to neuropsychiatric disorders such as anxiety (Shea, Walsh et al. 2005), depression (de Kloet, Joels et al. 2005, Pariante and Lightman 2008), ADHD (Ma, Chen et al. 2011), and learning/memory deficits (Lupien, de Leon et al. 1998, Finsterwald and Alberini 2014, Wirth 2015). This raises the question as to whether prenatal oxycodone exposure affects behavior of the offspring. In fact, both human and animal studies have shown that exposure to opiates has deleterious effects on neurodevelopmental outcomes. However, most of the previous studies were using other opiates, including morphine and heroin rather than oxycodone. With illegal oxycodone use during pregnancy on the rise, it is important to study the effects of perinatal oxycodone exposure on the neurodevelopmental outcome of the offspring. In addition, these studies are necessary because the effects of opiates on the HPA-axis differ based on the paradigm and the type of opiate. We conducted this study using three testing paradigms. We used isolation-induced ultrasonic vocalizations (USV) to study the response of young rat pups
when separated from their mothers (Adams, Miller et al. 1983, Branchi, Santucci et al. 2001). USVs are considered an adaptive response and the characteristics of these USVs have a direct correlation with distress and/or anxiety in the rat pup (Simola 2015). USVs elicit maternal behavior and play an important role in the interaction between the pup and the dam (Branchi, Santucci et al. 2001). USV cues may be comparable to the crying sounds of human infants, (Zeskind, McMurray et al. 2011) which have been used to identify infants at risk for poor neurobehavioral outcomes (Lester 1987, Zeskind, McMurray et al. 2011). Although existing data on the effects of prenatal opiate exposure on isolation-induced USVs remain limited, exposure to other illicit drugs, such as cocaine, can alter many USV characteristics associated with changes in maternal care (McMurray, Zeskind et al. 2013). Neonatal exposure to alcohol is also associated with USV deficits with longer latency to the first USV and fewer numbers of USVs (Wellmann, Lewis et al. 2010). Although the underlying pathways and neurotransmitters involved in USV are complex, USV deficits in female rat pups exposed neonatally to alcohol are reduced by the n-methyl-d-aspartate receptor (NMDAR) modulator agmatine (AG). AG reduces glutamate release and/or blocks the polyamine modulatory site on NMDAR (Gibson, Harris et al. 2002, Feng, LeBlanc et al. 2005), therefore suggesting that the increased NMDAR activities resulted from neonatal ethanol exposure (Wellmann, Lewis et al. 2010). Interestingly, not only prenatal alcohol exposure (Naassila and Daoust 2002) but also opioid exposure/opiate withdrawal (Zhang, Liu et al. 2016) and prenatal exposure to morphine (Yang, Yang et al. 2000, Yang, Huang et al. 2003) are reported to alter NMDAR activities and properties in multiple brain regions. Although currently there are no published data, we may postulate that prenatal oxycodone exposure may increase NMDAR expression or activity which in turn may decrease the USV response. Thus, we hypothesized that rat pups prenatally exposed to oxycodone will have
increased latency to the first vocalization and decreased numbers of vocalizations per
minute during USV testing compared to controls.

Although animal data showed that prenatal exposure to morphine was not
associated with increased locomotor activity in the open field (Slamberova and Vathy
2002, Buisman-Pijlman, Gerrits et al. 2009), human studies revealed that children
exposed to opiates in utero were found to have hyperactivity, impulsivity, and attention
problems, whether the mothers were in opiate maintenance therapy with buprenorphine
or the mothers were polysubstance-users (Walhovd, Moe et al. 2007, Sundelin Wahlsten
and Sarman 2013). Therefore, we hypothesized that POE is associated with hyperactive
behaviors in rat offspring when tested in the open field. The open field test has been
widely used to study hyperactivity, anxiety, and stress in animals (Tou and Wade 2002,

Good evidence indicates that children prenatally exposed to opiates have
learning problems and lower scores on neurodevelopmental tests compared to control
un-exposed groups (Guo, Spencer et al. 1994, Bunikowski, Grimmer et al. 1998, Hunt,
Tzioumi et al. 2008). Animal studies also have shown that prenatal exposure to
morphine is associated with learning and memory deficits in the offspring (Niu, Cao et al.
2009, Davis, Franklin et al. 2010). Thus, we hypothesized that POE will impair learning
and memory of the offspring as tested in the water maze.
7.2 Material and Methods

7.2.1 Experimental Design: Animals and Prenatal Treatments.

The rat dams and pups were treated as described in Chapter 4. At 5 pm on PD 5, all pups in each litter were transferred to an untreated foster dam to minimize exposure to altered maternal rearing behavior that has been described in rat dams after exposure to opiates during pregnancy (Slamberova, Bar et al. 2003). To preclude potential litter effects, a maximum of one male and one female from each litter was included in the behavioral studies (Chapman and Stern 1979).

7.2.2 Experimental Design: Neurobehavioral Tests

All the behavioral tests were performed in collaboration with Dr. Susan Barron’s laboratory.

7.2.2.1 Ultrasonic Vocalizations (USV)

USVs were examined according to published procedures (Barron and Gilbertson 2005). In brief, an ultrasonic bat detector (Ultra Sound Advice Model #S-25, UK- http://www.ultrasoundadvice.co.uk) set at 40 kHz with a condenser microphone (SM-1) set 21.5 cm above the test cage floor was used. The output was recorded on a SONY #WM-D8C Cassette Recorder using low noise cassette tapes. On PD 14, the pups in each litter were separated from the dams, remaining in their home cage and were brought to a neighboring test room one litter at a time. Their cages were placed halfway on a heating pad to provide warmth. During testing, the dam was placed in a new cage in the same test room. CON offspring (n = 6) and OXY-L (n = 4) and OXY-H (n = 8) rats underwent USV testing. During USV testing, pups were isolated one at a time in a clean cage for 6 minutes during which USVs from the pup were recorded. A fan was used to provide white noise during testing. Upon completion of USV testing for the entire litter,
the dam was returned to the home cage and the home cage was returned to their original animal room.

Assessment of USV: Audio data for each 6-minute test period were individually scored for latency to vocalization (seconds) and numbers of USVs per minute per test period. Scoring was performed independently by 2 experimenters who were blind to the treatment groups and used a stopwatch and clicker counter. Similarity of their scores was compared and indicated that the reliability between experimenters was greater than 90%.

7.2.2.2 Open Field Test

On PD 43, the offspring were transferred to another building, where they were housed for at least 7 days prior to subsequent behavioral testing. The open field test was performed first and was preceded by 5 days of habituation followed by 2 days of 3-minute handling periods which finished with weighing. CON offspring (n = 30, 19 males, 11 females) and OXY-L (n = 8, 4 males, 4 females) and OXY-H (n = 32, 17 males, 15 females) rats underwent open field tests on PD 50-51. The number of pups in the OXY-L group was smaller due to limited numbers available after other experiments were performed. The open field apparatus was a circular field 36 cm tall and 58 cm in diameter designed to prevent thigmotaxis or excessive time in corners (Lewis, Wellmann et al. 2012). In addition, the open field was divided into two zones, with the center zone representing 25% of the total area and the outer zone representing 75% of the total area. All testing was conducted and housed in a test room with white noise to reduce external distractions. On day 1 of testing, two rats were transferred in separate cages into the test room for a 10 minute habituation period. Each animal was then placed in a separate open field and their activities were recorded with a Polytracker® (San Diego Instruments, San Diego, CA) for 30 minutes. The animals were then returned to their
home cages in the colony room upon completion of testing. Activity was measured on two consecutive days.

Assessment of open field activity: The recorded activity of each rat was assessed in 5 minute blocks of time across the 30 minute test period for each day. Total distance traveled (centimeter, cm), distance traveled in the center zone, and the ratio of distance traveled in the center zone (middle 25% of the open field) to total distance traveled or to outer zone were determined.

7.2.2.3 Water Maze

On PD 55-56, two groups of offspring, CON (n = 20, 11 males, 9 females) and OXY-H (n = 20, 10 males, 10 females) were tested in the water maze. Rats from OXY-L group were not tested due to the limited numbers of pup from this group that were available after other experiments (described in previous chapters) were performed. This experiment was modified from a procedure previously described (Lewis, Wellmann et al. 2012). The apparatus was a 130×90×40 cm black Plexiglas chamber, divided such that several divergent paths, each 18 cm wide, branched off from the central start area. The apparatus and methodology were modified from von Euler et al. (2006) (von Euler, Bendel et al. 2006). Water temperature was maintained at 76°± 2. In this test, rats must learn to swim and make three successive right/left choices to a platform that is submerged below the water level and invisible; the water was made black with the addition of nontoxic black tempura paint to obscure the submerged platform. A plastic sheet surrounded the maze, reducing extra-maze cues, including the experimenter. A major advantage of this maze is that control animals can learn the maze in a single day. Movement in the maze was recorded using a video tracking system (SMART program; Panlab, S.L.) (Lewis, Wellmann et al. 2012).
On the first trial, a rat was placed in the maze and allowed to swim freely. If the animal did not reach the platform after 1 minute, it was guided to the platform. After 5 seconds on the platform, the animal was transferred to a cage warmed by heated lamp (25 W) for 30 seconds. Trials were repeated until the animal reached criterion performance, defined as completion of 2 consecutive trials without making any errors (wrong turns) or a total of 3 successful trials. The number of trials the animal took until criterion performance was reached was recorded as the outcome measure. The next day the rats were tested for retention using an identical procedure.

7.2.3 Statistical Analyses

Statistical analyses were considered significant if p<0.05. Multivariate linear regression models with random litter effect (or Gaussian linear model with compound symmetry covariance structure) were used to analyze USVs, OF and Water Maze data. The models were fitted using restricted maximum likelihood to test for the impact of treatment group, gender, time, and any potential interactions these variables have on the mean values for the data. Due to skewness and outliers of the data, a natural log transformation was applied when appropriate. The Kenward and Roger (1997) approximation was used to appropriately estimate standard error and degrees of freedom. Variables (trial days, sex and treatment groups) were treated as categorical and backward elimination at the 5% significance level was utilized. Kruskal-Wallis tests were applied to compare litter sizes and number of males per litter between treatment groups. A linear mixed effects model was applied to compare differences in body weight between groups with treatment group, gender, PND and the interaction of gender and PND included as predictors of weight. Tests were two-sided and were conducted in SAS version 9.4 (SAS Institute, Cary, N.C.)
7.3 Results

7.3.1 Litter Size and Body Weight

Litter size did not differ as a function of prenatal oxycodone exposure (Table 7.1). There were also no significant differences in the number of males and females per litter as a function of prenatal treatment (Table 7.1).

Although the means of maternal weight gain during pregnancy were different between groups (p=0.005), drug treatment did not affect mean birth weight of both male and female pups (table 7.1). The pups were weighed within 24 h after birth. Rat dams in OXY-H group displayed less weight gain compared to the CON dams (mean difference 39.65 g, p = 0.01) and compared to the dams in OXY-L group (mean difference 42.75 g, p = 0.023) (table 7.1). Using a linear mixed model, drug treatment did not affect weight gain of the pups; body weights from birth up to PD 45 were not significantly different between groups (p = 0.61). However, there was a significant interaction between gender and postnatal age (P < 0.0001); male and female had different growth trajectories over time (Figure 7.1a and 7.1b) as expected.

Body weights of both male and females were similar across treatment groups in the rats that were tested in the open field and water maze (p = 0.43). The females weighed less than the males as expected (p<0.0001) (table 7.1).

7.3.2 USV

There were no significant differences between males and females so results from both genders were combined for USV subsequent analyses. There was no significant difference in the latency to the first vocalization between treatment groups, however, there was a trend for it to be longer in OXY-exposed rats (Figure 7.2 a). Latency to the first USV as plotted in Kaplan –Meier Plots is shown in Figure 7.2b, a point on the plot
denotes the estimated probability of having a first USV after the given time point.
Although there was a trend for prenatal oxycodone-exposed pups to display longer
latencies to their first USV after isolation, there were no significant differences between
treatment groups (p=0.25), likely due to small sample sizes (Figure 7.2b).

Total USVs across time also did not differ among the 3 treatment groups
(p=0.85) (Figure 7.2c). The number of USVs increased across time in all treatment
groups (p = 0.0004); however, there was no significant effect of perinatal OXY exposure
(p = 0.32) (Figure 7.2d).

7.3.3 Open Field Test

Analysis of total distance traveled in 30 minutes

Analysis of the raw data indicated that there were no statistically significant
differences in total distance traveled among rats in CON, OXY-L or OXY-H groups
(p=0.26). There was also no effect of test day on the total distance traveled (p=0.48). No
interactions were significant. Female rats traveled a greater total distance compared to
males [traveled 1654 cm further than males, 95% CI: (539, 2768), p = 0.004] (Figure
7.3a). However, when a natural log transformation was applied due to skewness and
outliers of the data, both treatment group and gender had significant effects (p=0.002
and p<0.0001 respectively). Namely, rats in OXY-L group traveled a greater total
distance compared to the other treatment groups (vs OXY-H, p=0.011; vs CON, p =
0.0004, Figure 7.3a). No interaction between gender in OXY-L group was detected in the
small sample size. Male rats had a lower mean total distance traveled compared to
females (p<0.0001).

Using a linear mixed model, the analysis of the total distance traveled in each 5
minute block showed that there was no significant main effect of perinatal treatment or
test day (p = 0.10 and p = 0.07 respectively). However, the statistical model estimated
that females travel 241 cm further than males in any given 5 minute period (95% CI: (72,410), p = 0.003). The mean total in each 5 minute time block significantly decreased over testing time as expected (p < 0.0001) (Figure 7.3b).

**Analysis of total distance traveled in the inner zone, outer zone and mean ratio of distance traveled in the inner/outer zone**

**The Inner Zone**

There was a significant main effect for perinatal treatment (p = 0.011) and gender (p = 0.007). Rats exposed to OXY-L traveled more in the inner zone than rats in the other 2 treatment groups (OXY-L vs OXY-H, p = 0.011; vs CON, p = 0.003, Figure 7.3c). This difference was likely largely driven by OXY-L-treated males on day 2 (Figure 7.3c). The distance traveled in the inner zone did not differ between days (p = 0.61) and no interactions were significant.

The analysis of the distance traveled in the inner zone in each 5 minute time block showed that there were no group differences in distance traveled in the inner zone across time (Figure 7.3d). The large standard errors were due to the small sample size.

**Outer Zone**

There were no significant interactions. Perinatal treatment or testing day did not impact the mean total distance traveled in the outer zone (p = 0.35 and p = 0.29 respectively) (Figure 7.3e). When natural log transformation was applied and 2 outliers were removed, there were significant differences between gender (p < 0.0001) and treatment group (p = 0.005). Namely, males traveled to the outer zone less than females (p=0.003) and OXY-L rats traveled more in the outer zone than the CON group (p = 0.002, Figure 7.3e). There was no difference between CON or OXY-L vs OXY-H (Figure 7.3e).

The analysis of the distance traveled in the outer zone across the 5 minute blocks showed that there were no statistically significant interactions. There was no
effect of perinatal treatment ($p = 0.15$). The model estimated that females travel 194 cm further than males in the outer zone in any given 5 minute period [95% CI: (49, 339), $p = 0.01$] (Figure 7.3f). In addition, rats tended to travel 113 cm further on the first test day compared to the second day in any given 5 minute time period [95% CI: (10, 216), $p = 0.032$] (Figure 7.3f). The mean total distance traveled in the outer zone in a 5 minute period significantly decreased over time as expected ($p < 0.0001$) (Figure 7.3f).

The Ratio of the Distance Traveled in the Inner Zone to Outer Zone

The ratio of activity in the center zone vs outer zone of the field is a potential marker of motor impulsivity and/or anxiety (Royce 1977). There were no differences in mean ratios across test days ($p = 0.18$) but there was a statistically significant treatment group x sex interaction ($p = 0.003$; sex x treatment group interaction). Specifically, the estimated mean ratio of the distance traveled in the inner to outer zone of male OXY-L rats which was 0.245, was statistically calculated to be 0.095 larger than that of the CON [0.14; 95% CI for CON: (0.027, 0.162), $p = 0.008$], and 0.112 larger than for OXY-H [0.12; 95% CI: (0.042, 0.1830, $p = 0.002$] (Table 7.3). This pattern differed across sex; no differences in the inner:outer ratio were observed in the females (estimated mean ± SEM for days 1 & 2 in CON: 0.13 ± 0.01, OXY-L 0.1 ± 0.05 and OXY-H 0.17 ± 0.02) (Table 7.3). These differences in the male persisted when the ratios of distance traveled in the inner to outer zone for each 5 minute time block were analyzed (OXY-L vs CON: $p = 0.04$; OXY-L vs OXY-H = 0.007) (Figure 7.3g).

7.3.4 Water Maze

Using a multivariate repeated measures Gaussian linear model to analyze the data with trial days, treatment groups and sex as predictor categories, there was no effect of oxycodone or sex on the means of the average number of trials until criterion performance was reached on both day 1 and day 2 ($p=0.62$). The expected decrease in
the means of the average number of trials on day 2 was observed in all groups (p<0.0001) (Figure 7.3h).

7.4 Discussion

In this experiment we explored whether perinatal oxycodone exposure had effects on behavioral outcomes using three different behavioral tests (USV, OF, and WM) in different age ranges. We found significant effects of POE on locomotion and preference for the center in the OF test consistent with hyperactivity. However, we did not find any effects of POE on the adaptive response of the rat pups when separated from the mothers during the USV test, or on learning and memory in the water maze.

USV

For the USV, our results showed that although there was a trend toward increased latency to first vocalization, we did not find any statistically significant main effects or interactions. The number of vocalizations per minute was also not significantly different between the treatment groups. There are several possible reasons why we did not detect differences in these USVs parameters. First, our low dose oxycodone had a relatively small sample size. Based on the current data, we may be able to detect the effect on oxycodone on the latency to first vocalization if we increase the sample size to 9 per group. Even though the data on the effects of prenatal oxycodone or opiate exposure on the USVs are limited, perinatal exposure to other substances of abuse such as cocaine (Cox, Hodge et al. 2012) and alcohol (Rubin, Wellmann et al. 2009), and perinatal insults such as hypoxia (Saucier, Ehresman et al. 2008) have been described to have effects on USVs. In addition, these effects seem to be age- and sex-dependent which correlate with the limbic circuitry development, as evidenced in the prenatal
cocaine exposure model (Cox, Hodge et al. 2012). Specifically, the numbers of USVs on PD1 are decreased in rats prenatally exposed to cocaine, the period before the emotional controls of USVs are fully developed. The differences in USV parameters can also be detected again on PD 21 when emotional control of the USVs has begun to emerge, but not on PD 14 when a peak vocalizing of all offspring occurs (Cox, Hodge et al. 2012). Therefore, it is also possible that we did not find differences in any parameters of the USVs when testing was limited only to PD 14. This possibility has to take into account that the underlying mechanisms for various drugs involving USV are likely different since the effects of other substances such as alcohol on the USVs have been demonstrated on postnatal day 15 (Rubin, Wellmann et al. 2009).

Open Field Test

Rats exposed to oxycodone at a low dose were hyperactive and traveled more distance in the inner zone in the open field test compared to CON and OXY-H, which also correlated with a significantly higher mean ratio of the distance traveled in the inner zone to outer zone in the male OXY-L rat. These results indicate that rats prenatally exposed to oxycodone develop hyperactive behavior that is present through adolescence. In addition, the increase in the ratio of distance traveled in the inner zone to the total distance traveled could suggest that prenatal exposure to oxycodone reduced anxiety-like behavior and decreased normal species-typical thigmotaxic behavior when rats were placed in the new environment. Alternatively, these differences might just be the effects of an overall hyperactivity with a failure to inhibit entries to the center. In future studies, we can plan to differentiate whether the increased distance in the center was due to deficits in hyperactivity, impulse control, or reduction in anxiety.
Hyperactivity and increased locomotion in animals have been linked to Attention Deficit Hyperactivity Disorder (ADHD) (Viggiano, Ruocco et al. 2004), which has inattentive, and hyperactive or impulsive symptoms as hallmarks (Thapar and Cooper 2015). Our preliminary finding, which suggests that perinatal exposure to oxycodone is associated with hyperactivity in the offspring, is in agreement with human studies that identify hyperactivity, impulsivity, and attention problems in children exposed to opiates in utero (Walhovd, Moe et al. 2007, Sundelin Wahlsten and Sarman 2013). In animal models, however, the results are more conflicting. Therefore it is difficult to compare our results to others due to different studied drugs, which were mainly morphine, as well as different paradigms, age of testing, instruments used, and outcome measures. There are many inconsistencies in the literature as to how prenatal opiate and/or stress exposure alter the behavior of the offspring in the open field. A study by Slamberova et al showed that postnatal handling but not prenatal morphine exposure increased locomotor activities in the OF in adult male and female rats (Slamberova and Vathy 2002). In accordance with Buisman-Pijlman et al, it was shown by others that postnatal stress but not prenatal exposure to morphine increased locomotor activity in the OF (Buisman-Pijlman, Gerrits et al. 2009). Some investigators have reported that prenatal exposure to morphine was associated with increased anxiety like behavior in an elevated plus maze (EPM) and reduction in time spent in the lit side of the light/dark box (L/D box) (Ahmadalipour, Sadeghzadeh et al. 2015). Others found that rats prenatally exposed to morphine exhibited decreased anxiety-like behavior in the EPM and L/D box with no differences in the distance traveled over 30 mins in the OF (Tan, Duan et al. 2015). Klausz et al also found that there was no significant anxiogenic effect of prenatal morphine exposure detected in the EPM (Klausz, Pinter et al. 2011). Prenatal exposure to opiates, such as methadone, buprenorphine, and worst of all morphine, increased
anxiety-like behaviors compared to control groups in the light-dark transition test, with no effect on locomotor activity in an OF (Chen, Chiang et al. 2015).

A possible mechanism by which prenatal exposure to opiates could result in hyperactivity may involve changes in multiple neurotransmitter signaling pathways such as dopaminergic pathways and the HPA system. It is well described that opioids have a significant role in controlling the release of dopamine and acetylcholine in the key reward regions of the brain including the ventral tegmental area (VTA) and the nucleus accumbens (NA) (Gysling and Wang 1983, Rada, Barson et al. 2010, Jhou, Xu et al. 2012, Chartoff and Connery 2014). Long term exposure to opiates leads to both structural and biochemical changes in the mesolimbic dopaminergic system; for example, a reduction in the cell size of dopaminergic neurons in the VTA (Sklair-Tavron, Shi et al. 1996), and increased levels of tyrosine hydroxylase, which is the rate limiting enzyme in the synthesis of dopamine in the VTA (Beitner-Johnson and Nestler 1991). In addition, convincing evidence suggests that the impairment of dopamine-mediated development and the monitoring of motivated behavior and reward-related memory formation might be associated with ADHD symptoms (Sagvolden, Johansen et al. 2005, Johansen, Killeen et al. 2009). Thus, taken together, it is possible that perinatal exposure to opiates may disrupt the normal development of dopaminergic reward-related circuits leading to hyperactive behavior. However, this speculation remains to be further elucidated.

Changes in the HPA system have also been linked to ADHD symptoms. In children, both reduced basal cortisol secretion and cortisol hyporeactivity have been associated with hyperactivity/impulsivity or a combined type ADHD (Fairchild 2010). An abnormal diurnal rhythm and less effective negative feedback mechanisms after a dexamethasone suppression test were also identified more frequently in the children
with ADHD that were severely hyperactive compared to the children with milder symptoms (Kaneko, Hoshino et al. 1993). Interestingly, it was demonstrated recently in adults that, although the basal salivary cortisol levels were not different, cortisol levels 20-min after a mental cognitive stress test in adults with ADHD were significantly higher compared to those in healthy adult controls (Raz and Leykin 2015). These findings are in line with our findings that POE is associated with increased corticosterone reactivity to the restraint stress test in adult female rats. This was likely in part due to the impaired negative feedback mechanisms and increased feedforward stimulation as described in Chapters 4 and 5. In addition, we found that the hyperactivity was more notable in the male OXY-L group, which is in agreement with many community-based studies that have shown that ADHD is more prevalent in the males (Thapar and Cooper 2015). However, we have found that the differences in the CORT response to the RST were actually in the female, and these differences can’t explain the hyperactive symptoms in this experiment. Raz et al studied cortisol levels in individuals with ADHD and in a control group during an experimentally-induced psychological test; although they were able to detect differences in cortisol levels between the ADHD and control groups, they were not able to detect an interaction between genders (Raz and Leykin 2015). In fact, that we did not measure CORT during the behavioral tests could be a limiting factor. In addition, this discrepancy between hyperactivity in the males and increased levels of corticosteroids in the females could be due to various reasons, for example, the methods used to produce the stress in each study, relatively small sample sizes, and the gender of the participants, i.e., the majority were female in the study by Raz et al. Referral biases and methodological difficulties were present in other human studies (Biederman, Kwon et al. 2005, Williamson and Johnston 2015). Thus, the relationship between perinatal oxycodone exposure, hyperactive behaviors, and abnormal HPA-axis function that may interact with gender warrants further investigation. This may include the
measurement of CORT after neurobehavioral testing in our future experiments. The abnormal HPA-axis response to stress may be an interesting candidate to be used as a biomarker for early detection and treatment of ADHD in infants prenatally exposed to oxycodone/opiates.

Data from both animal and human studies suggest that early nutritional stress and malnourishment is associated with anxiety, high impulsivity, and attention problems (Lukas and Campbell 2000). Although the weight gain of the dams in OXY-H group was significantly lower than in those in the other groups, the weight gain of the dams in OXY-L group was actually comparable to that in the CON group. In addition, birth weights of the pups were comparable in all groups. Therefore, the hyperactivity in the OF of the OXY-L group could not solely be explained by poor maternal nutritional status.

**Water Maze**

We did not find the effects of perinatal oxycodone exposure on spatial learning and/or memory in the water maze test as hypothesized; this may have resulted from not including rats perinatally exposed to a lower dose of oxycodone in this experiment due to the small sample size of this group. Previous studies using prenatal morphine exposure models show conflicting data; memory and learning in rodents are either impaired or enhanced. Namely, juvenile rats prenatally exposed to morphine had impaired spatial memory in the Morris water maze or Y-maze test (Yang, Huang et al. 2003, Yang, Liu et al. 2006, Niu, Cao et al. 2009), but on the other hand, aberrant memories such as morphine reward memory in the conditioned place preference or forced swim tests were enhanced (Gagin, Kook et al. 1997, Wu, Chen et al. 2009, He, Bao et al. 2010, Klausz, Pinter et al. 2011). In contrast to our study, Davis et al (Davis, Franklin et al. 2010) found that prenatal exposure to oxycodone impaired spatial learning and memory in a battery
of spatial tasks; in the Morris water maze, rats prenatally exposed to OXY had increased latency and greater distance traveled to find the platform when the intertrial interval was long, not short. Rats prenatally exposed to oxycodone also had a decreased use in spatial strategies and more use of non-spatial strategies such as wall-hugging. In addition, the retention of learning memory in the T-maze, assessed 5 days after acquisition of the training, was impaired. This finding is actually consistent with ours, as described in Chapter 6, that POE male rats had impairment in retention of the discrimination ability when retested on PD 75, although they were able to correctly discriminate between the stress and non-stress cues during the classical conditioning paradigm on PD 40. Moreover, rats prenatally exposed to oxycodone have more reference memory errors in the radial arm maze (Davis, Franklin et al. 2010). The differences in the results from Davis et al compared to ours could be due to many reasons. In their study, rat dams were treated with different OXY paradigms including the dose and route of administration of oxycodone; rats in the Davis et al study received escalating doses of OXY (10 mg/kg/day up to 15 mg/kg/day) via gavage for 28 days before harem breeding occurred. Importantly, the pups were reared by their biological mothers in their study while we fostered the pups. It had been shown by many studies that exogenous opiate administration negatively alters maternal rearing behavior such as cleaning of the pups, delay of maternal behaviors, and maternal aversion to the odor of the pups (Bridges and Grimm 1982, Mayer, Faris et al. 1985, Kinsley, Morse et al. 1995). Neonatal rearing condition and neonatal maternal interaction such as maternal separation had long term effects on the stress response of the offspring including an increase in restraint stress-induced norepinephrine release in the PVN in adult rats (Liu, Caldji et al. 2000), and changes in the HPA-axis at multiple levels that could be linked to epigenetic modification (Sng and Meaney 2009). Variations of maternal care also influence learning and behaviors of the offspring (Menard and Hakvoort 2007, Lindeyer,
Meaney et al. 2013). The adverse effects of neonatal maternal separation on the HPA axis were lessened by fostering the litters as had been shown by Huot et al (Huot, Gonzalez et al. 2004). Thus, fostering the pups when the dams were exposed to opiates could alter the HPA-axis and possibly the neurobehavioral outcomes of the offspring. In addition, even though signs of withdrawal in the dams were monitored in the study by Davis et al, body weights of the OXY pups were approximately 10% lower than those of the controls, indicating possible neonatal opiate withdrawal and poorer nutritional status that can also affect long term outcomes; however, the body weights of the pups in our study were comparable in all groups.

Studies in humans showed that prenatal exposure to opiates resulted in impairments in cognitive function and learning. Bunikowski et al also explained that when evaluated at one year of age, children prenatally exposed to opiates had a mild psychomotor developmental impairment compared to the control group; these included impairments in "hearing and speech" and "intellectual performance" subscales (Bunikowski, Grimmer et al. 1998). Guo et al found that in utero opiate exposure was associated with impairments in the Auditory Rare Event Monitoring (AREM) task and the Sternberg Memory task in children 7-12 years of age (Guo, Spencer et al. 1994). More recently, Hunt et al reported from their case-control study that infants prenatally exposed to opiates were more likely to experience neurodevelopmental impairments compared to healthy control infants, when assessed at 18 months and 3 years of age (Hunt, Tzioumi et al. 2008). The deleterious effects of prenatal opiate exposure on cognitive function persisted and did not decrease over time after controlling for permanent home placement and heroin use in the mother when children prenatally exposed to opiates were retested on the Wechsler Intelligence Scale for Children from 1 year old up to 8.5 years of age (Nygaard, Moe et al. 2015). These data suggested that prenatal exposure
to opiates is associated with impaired cognitive functions. Therefore, although we did not detect the effects of POE on cognitive function, learning, and memory using a water maze as a paradigm, further investigation is warranted. We could use different testing paradigms in lieu of a water maze or test the retention of memory after a longer period of time and include rats exposed to a lower dose of oxycodone in our next study.

Interestingly, we found that exposure to the lower dose of oxycodone of 0.5 mg/kg/day but not the higher dose of 2.0 mg/kg/day was associated with hyperactivity in the offspring. This could possibly be due to the development of tolerance in the rat dams that received higher doses of oxycodone as explained in Chapter 4. Opiate tolerance is characterized to be pharmacodynamic, time and dose-dependent, and opioid receptor specific (Collett 1998). Although more extensively studied following exposure to a mu opioid receptor agonist (MOR), Iyengar and Kim et al described that tolerance can develop after exposure to a kappa opioid receptor (KOR) agonist (Iyengar, Kim et al. 1987) even for as short as five days (Ignar and Kuhn 1990). Whether or not tolerance to the stimulatory effects on the HPA-axis by KOR agonists is dose-dependent is not well studied. But tolerance to the MOR agonist had been shown to be dose-related (el Daly 1996). Taken together, it is possible that in our study, the rat dams that were exposed to the higher dose of oxycodone (2 mg/kg/day) may have developed opioid tolerance leading to a decreased fetal CORT exposure, thus there were less effects on the developing HPA-axis and the neuro-developmental outcomes of the offspring.

In summary, POE was associated with hyperactivity in adult rats. Although we did not detect changes in responses when separated from the dams or in learning and memory deficits, this could be due to our small sample sizes, testing paradigms, or not including the rats exposed to the lower dose of oxycodone in the WM test. However, we found memory retention problems in POE rats as described in Chapter 6. We can
propose for our next study to increase the sample size particularly to investigate the neonatal stress response during USV and to further explore the relationship between hyperactivity, the HPA axis dysfunction, and gender interactions.
Table 7.1: Litter Size, the Number of Male and Female Rat Pups per Litter, Maternal Weight Gain during Pregnancy and Birth Weight of the Pups

<table>
<thead>
<tr>
<th>Variable (number of litter)</th>
<th>CON (N = 11)</th>
<th>OXY-H (N = 13)</th>
<th>OXY-L (N = 5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of pups per litter</td>
<td>13.3 (14) ± 2.9</td>
<td>12.3 (13) ± 4.4</td>
<td>14.4 (14) ± 1.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Number of females per litter</td>
<td>5.9 (6) ± 2.1</td>
<td>6.5 (7) ± 3.1</td>
<td>6.0 (6) ± 1.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Number of males per litter</td>
<td>7.4 (8) ± 3.0</td>
<td>5.8 (6) ± 2.5</td>
<td>8.4 (8) ± 0.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Maternal weight gain during pregnancy Mean ± SEM (g)</td>
<td>153 ± 7.3*</td>
<td>113.35 ± 10*#</td>
<td>156.1 ± 9.0#</td>
<td>*p =0.01, CON vs OXY-H, diff 39.7 g #p= 0.023, OXY-H vs OXY-L, diff 42.8 g</td>
</tr>
<tr>
<td>Birth weight mean ± SEM (g)</td>
<td>M: 6.4 ± 0.2 F: 6.0 ± 0.2</td>
<td>M: 6.25 ± 0.3 F: 5.83 ± 0.3</td>
<td>M: 6.1 ± 0.4 F: 5.6 ± 0.5</td>
<td>n.s. n.s.</td>
</tr>
<tr>
<td>Body weight during neurobehavioral tests</td>
<td>M: 223 ± 6.7 F: 169 ± 5.4</td>
<td>M: 250 ± 1.5 F: 177 ± 3.5</td>
<td>M: 210 ± 8.0 F: 175 ± 4.9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Figure 7.1a: Body weight of Male Offspring

Body weight of male pups from birth through PD 50 in control (CON, closed circles, n = 81), oxycodone low dose (OXY-L, open circles, n = 42) and oxycodone high dose (OXY-H, closed triangles, n= 76) groups.
Figure 7.1 b: Body weight of Female Offspring

Body weight of female pups from birth through PD 50 in control (CON, closed circles, n = 65), oxycodone low dose (OXY-L, open circles, n = 30) and oxycodone high dose (OXY-H, closed triangles, n = 84) groups.
Figure 7.2a: Latency to the fist vocalization of rat pups during USV test in control (CON, white bar; n = 6), oxycodone low dose (OXY-L, gray bar; n = 4) and oxycodone high dose (OXY-H, black bar; n = 8) groups.
Figure 7.2b: Latency to the first USV as plotted in Kaplan–Meier Plots in rat pups in control (CON, solid line), oxycodone low dose (OXY-L, long dashed line) and oxycodone high dose (OXY-H, dotted line) groups.
Figure 7.2c: Total numbers of vocalization of rat pups during USV test in control (CON, white bar; n = 6), oxycodone low dose (OXY-L, gray bar; n = 4) and oxycodone high dose (OXY-H, black bar; n = 8) groups.
Figure 7.2d: The numbers of USVs per minute across the testing period in control (CON, closed circles), oxycodone low dose (OXY-L, open circles) and oxycodone high dose (OXY-H, closed triangles) group.
Figure 7.3a: Total distance traveled in 30 minutes in the OF test in male (left panel) and female offspring in control (CON, white bars; male n = 19, female n = 11), oxycodone low dose (OXY-L, gray bars; male n = 4, female n = 4) and oxycodone high dose (OXY-H, black bars; male n = 17, female n = 15) groups. Rats in OXY-L group had greater total distance traveled compared to the other treatment groups (vs OXY-H, p=0.011; vs CON, p = 0.0004) and male rats had lower mean total distance traveled compared to females (p<0.0001).
Figure 7.3b: Total distance traveled in each 5 minute time block in the OF test on test day 1 (upper panel) and test day 2 (lower panel), male (left panel) and female (right panel) in control (CON, closed circles), oxycodone low dose (OXY-L, open circles) and oxycodone high dose (OXY-H, closed triangles) groups.
Figure 7.3c: Total distance traveled in inner zone in 30 minutes in the OF test in male (left panel) and female offspring in control (CON, white bars), oxycodone low dose (OXY-L, gray bars) and oxycodone high dose (OXY-H, black bars) groups. There were significant differences between group (p = 0.011) and gender (p = 0.007). Rats exposed to OXY-L traveled more in the inner zone than rats in the other 2 treatment groups (OXY-L vs OXY-H, p = 0.011; vs CON, p = 0.003). No significant interaction was detected.
Figure 7.3d: Total distance traveled in inner zone in each 5 minute time block in the OF test on test day 1 (upper panel) and test day 2 (lower panel), male (left panel) and female (right panel) in control (CON, closed circles), oxycodone low dose (OXY-L, open circles) and oxycodone high dose (OXY-H, closed triangles) groups.
Figure 7.3e: Total distance traveled in outer zone in 30 minutes in the OF test, by male (left panel) and female offspring in control (CON, white bars), oxycodone low dose (OXY-L, gray bars) and oxycodone high dose (OXY-H, black bars) groups. The males traveled to the outer zone less than females ($p = 0.003$) and OXY-L rats traveled more in the outer zone than the CON group ($p = 0.002$).
Figure 7.3f: Total distance traveled in outer zone in each 5 minute time block in the OF test on test day 1 (upper panel) and test day 2 (lower panel), by male (left panel) and female (right panel) rats in control (CON, closed circles), oxycodone low dose (OXY-L, open circles) and oxycodone high dose (OXY-H, closed triangles) groups.
Table 7.3: The Mean Ratios of the Distance Traveled in the Inner to Outer Zone in Male and Female Rats in 30 Minutes Testing Period

### Male

<table>
<thead>
<tr>
<th>Test Day</th>
<th>CON (SEM)</th>
<th>OXY-L (SEM)</th>
<th>OXY-H (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.13 (0.01)</td>
<td>0.19 (0.09)</td>
<td>0.13 (0.01)</td>
</tr>
<tr>
<td>2</td>
<td>0.15 (0.01)</td>
<td>0.27 (0.07)</td>
<td>0.13 (0.01)</td>
</tr>
<tr>
<td>Estimated mean day 1&amp;2</td>
<td>0.14 (0.01)</td>
<td>0.245 (0.07)*</td>
<td>0.12 (0.01)</td>
</tr>
</tbody>
</table>

### Female

<table>
<thead>
<tr>
<th>Test Day</th>
<th>CON (SEM)</th>
<th>OXY-L (SEM)</th>
<th>OXY-H (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.13 (0.01)</td>
<td>0.10 (0.04)</td>
<td>0.17 (0.03)</td>
</tr>
<tr>
<td>2</td>
<td>0.14 (0.02)</td>
<td>0.11 (0.04)</td>
<td>0.17 (0.02)</td>
</tr>
<tr>
<td>Estimated mean day 1&amp;2</td>
<td>0.13 (0.01)</td>
<td>0.10 (0.05)</td>
<td>0.17 (0.02)</td>
</tr>
</tbody>
</table>

* p < 0.05 (the estimated mean ratio of distance traveled in the inner to outer zone of male OXY-L rats which was 0.245, was estimated to be 0.095 larger than that of the CON [95% CI: (0.027, 0.162), p = 0.008], and 0.112 larger than for OXY-H [95% CI: (0.042, 0.1830, p = 0.002)]
Figure 7.3g: The ratio of the distance traveled in the inner/outer zone in 30 minutes in the OF test (mean ± SEM) by male (left panel) and female (right panel) rats in control (CON, closed circles), oxycodone low dose (OXY-L, open circles) and oxycodone high dose (OXY-H, closed triangles) groups. Male OXY-L rats had overall higher ratios of distance traveled in the inner to outer zone for each 5 minute time block across time compared to other treatment groups (OXY-L vs CON: p = 0.04; OXY-L vs OXY-H = 0.007)
Figure 7.3h: The mean number of trials in the water maze in male (left panel) and female (right panel) rats in control (CON, white bars), and oxycodone high dose (OXY-H, black bars) groups.
The results from this dissertation demonstrate that perinatal oxycodone exposure (POE) has significant effects on the HPA-axis, cardiovascular response to stress and behaviors of the offspring that last to adulthood. Using two different strains of rats (Sprague Dawley and Dark Agouti), we demonstrate that offspring that were exposed to oxycodone by injections to the dam starting from gestational day 8 throughout the pregnancy and into the first week of postnatal age develop heightened HPA-axis responses to stress, including stress from a pharmacological challenge (CRH injection) or psychological stress (restraint stress test). These increased HPA responses may partly explain hyperactive behaviors in adult offspring exposed to oxycodone in utero. In addition, POE also leads to an increased blood pressure response to classical stress conditioning and impairs retention of memory in the offspring. Thus far, we have explored some possible mechanisms which may result in these changes in the HPA-axis response to stress. We demonstrate that POE increases the subpopulation of CRH neurons that also contain ERbeta immunoreactivity which then can exaggerate the stimulation of the HPA-axis. Moreover, POE decreases MR-mRNA expression in the hippocampus which can impair the negative feedback control of the HPA-axis by the limbic system. A summary diagram of these findings is depicted in Figure 8. We will next discuss how these changes in receptor density and gene expression in brain regions controlling the HPA axis may be programmed by epigenetic mechanisms, whether the effects from POE are modifiable by postnatal interventions, and how we may modify the model to better represent POE in humans in future studies. It is worth noting that beyond the mechanistic changes proposed in this dissertation, POE may lead
to changes in other elements of the HPA-axis which in turn, can modulate the development of the HPA-axis as previously shown in the prenatal stress models. These include changes in the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β–HSD2) in the placenta and in the fetal brain, which converts the glucocorticoids cortisol/corticosterone into the inactive metabolites cortisone/11β-dehydrocorticosterone (Jensen Pena, Monk et al. 2012), and the balance between the GR and MR expression (Zimmer and Spencer 2014). POE may also alter the expression of opioid receptors as previously shown in the prenatal morphine exposure model (Slamberova, Rimanoczy et al. 2003) and in the prenatal stress model (Sanchez, Milanes et al. 2000), which then may modulate the development and function of HPA-axis.

8.1 Does POE Lead to Epigenetic Changes in the Stress Axis?

Programming of the HPA-axis that can occur by a number of different environmental challenges during pregnancy and the postpartum period including stress, maternal care, nutrition, and possibly exposure to substances of abuse may be mediated by epigenetic mechanisms. Epigenetic mechanisms induce changes in the accessibility of transcription factors to specific genes leading to actively transcribed or silenced genes. These processes occur by either direct modification of the DNA through DNA-methylation at CpG islands in promoter or coding regions or modifications of histone protein by a process such as acetylation, phosphorylation, or methylation (Gräff and Mansuy 2008, Sananbenesi and Fischer 2009). A growing body of evidence indicates that epigenetic mechanisms are crucial processes by which the environment shapes brain development, leading to life-long alterations (Champagne and Curley 2009, Xie, Korkmaz et al. 2013, Bock, Rether et al. 2014, Lutz and Turecki 2014). Great examples
of how perinatal environment can modify the stress axis through epigenetic mechanisms are drawn from previous studies by Fish et al using a maternal care model (maternal high or low licking-grooming (LG), arch back nursing (ABN)) (Fish, Shahrokh et al. 2004). The studies show that high maternal LG of the rat pups programs the HPA-axis in such a way that they have a lower neuroendocrine response to stress, a more rapid return to baseline levels of CRH, ACTH, and CORT, better cognitive flexibility, and reduced anxiety during tests (Fish, Shahrokh et al. 2004). The pups in the increased LG-ABN group have an altered epigenome at the GR gene promoter in the hippocampus and have differences in DNA methylation, as compared to offspring of 'low-LG-ABN' mothers. These differences are associated with altered histone acetylation and transcription factor (NGFI-A) binding to the GR promoter (Weaver, Cervoni et al. 2004). Thus far limited data are available as to whether perinatal exposure to opiates/oxycodone leads to epigenetic changes in the HPA axis. We can propose to further study whether changes in the HPA-axis circuitry are associated with epigenetic changes in corresponding genes. These changes may include the hypomethylation of the ERbeta gene promoter in the PVN which in turn leads to increased ERbeta gene expression. In addition, hypermethylation of the MR gene promoter in the hippocampus, which may result in a decrease in MR gene expression, could occur.

Another mechanism by which environmental stressors epigenetically modulate gene expression is through post-transcriptional changes by miRNA expression (Conaco, Otto et al. 2006, Wiesen and Tomasi 2009, Uchida, Hara et al. 2010). MiRNAs are a class of short noncoding RNAs that play key roles in vital cellular processes, including how cells respond to changes in environment. MiRNA biogenesis starts in the nucleus and is followed by transfer to the cytoplasm, where it binds to the recognition element leading to its degradation or translational repression (Hollins and Cairns 2016). MiRNAs
are abundantly expressed in the brain and have diverse roles in development and other functions of the brain including neurogenesis (Maiorano and Mallamaci 2009), migration and differentiation (Sempere, Freemantle et al. 2004), myelination (Dugas, Cuellar et al. 2010), and adaptation to stress (Leung and Sharp 2010). Recent research suggests that prenatal stress, such as maternal anxiety, leads to alterations in miRNA expression which have been linked to inflammatory responses and bipolar disorder (Zucchi, Yao et al. 2013). Substances of abuse have also been shown to affect miRNA expression; prenatal alcohol exposure leads to changes, either increases or decreases in miRNA expression in fetal brain (Wang, Zhang et al. 2009, Laufer, Mantha et al. 2013). For example, out of 509 miRNAs screened, six miRNAs were upregulated and eight miRNAs were down regulated in the study by Wang et al (Wang, Zhang et al. 2009). However, no data are available to date that indicate whether prenatal exposure to opiate or oxycodone alters miRNA expression which may in turn alter the expression of genes involved in the HPA-axis circuitry. Therefore we can propose to further study whether changes in the HPA-axis circuitry are associated with epigenetic modification by miRNAs that will lead to changes in gene expression in the HPA-axis circuitry.

### 8.2 Are the Effects of POE on the Stress Axis Modifiable by Postnatal Intervention?

Although the evidence from previous studies has shown that epigenetic changes in the HPA-axis are stable and persist in adulthood, they seem to be susceptible to plasticity. Weaver et al demonstrated that the effects of maternal care on GR expression and the maternal effect on stress responses in the offspring can be reversed by central infusion of a histone deacetylase inhibitor (Weaver, Cervoni et al. 2004), suggesting a
causal relation and reversibility among epigenomic states. Moreover, Weaver et al also demonstrated that the infusion of methionine, the precursor of the methyl-donor S-adenosyl-methionine, which has been proposed to cause DNA hypermethylation by either activating DNA methylation enzymes or inhibiting active demethylation, reverses the effect of maternal behavior on epigenetic changes. These effects of maternal behavior include DNA methylation, nerve growth factor-inducible protein-A binding to the exon 1, promoter, GR expression, and HPA-axis and behavioral responses to stress (Weaver, Champagne et al. 2005). Epigenetic effects of miRNA have been shown also to be reversible by pharmacological intervention; folic acid supplements block the upregulation of miR-10a caused by prenatal alcohol exposure and prevent ethanol-induced teratogenesis (Wang, Zhang et al. 2009). Therefore, it is reasonable for us to suggest that epigenetic changes that may be caused by perinatal exposure to oxycodone are possibly reversible by postnatal interventions. In fact, recently published data demonstrate that environmental enrichment can reverse some of the epigenetic changes caused by adverse perinatal milieus (Wang, Nie et al. 2014, Gapp, Bohacek et al. 2016, Wu, Bie et al. 2016).

Although pharmacological interventions provide intriguing evidence for reversal of the epigenetic changes, their clinical applicability is questionable. Environmental enrichment, on the other hand, is of particular interest as it is more applicable in human settings and seems to have effects at many levels and sites. We can propose for our future experiments to provide environmental enrichment to offspring that are perinatally exposed to oxycodone and hypothesize that this intervention will reverse the epigenetic modifications. In turn, we might be able to reverse the changes that occur in the HPA-axis and neurobehaviors.
8.3 How to Improve POE Model

We have modified the model in the course of this dissertation to better control for confounders including fostering the pups to control for maternal behaviors and using different rat strains. However, certain limitations still exist; for example, the dams were not actually exposed to oxycodone prior to becoming pregnant as is the case in addicted pregnant women. The dams were subjected to the additional stress of daily handling and subcutaneous injections, which we have controlled through the control group that were exposed to the same kinds of stress. Regardless, we can propose to improve the model by using a programmable mini pump which can be implanted before mating. With this approach, the dams will receive oxycodone before becoming pregnant and the dose of oxycodone can be programmed to escalate throughout pregnancy. It will also prevent additional stress from daily handling and injection. We can propose to utilize this technique in our future studies. This model will also provide a platform for us to study the effects of other substances of abuse on developing brains. In addition, the stages of the estrous cycle modulate the response of the HPA-axis; we propose to monitor the estrous cycle by performing vaginal lavage in the offspring in our future studies.

8.4 Conclusion and Summary

Previous studies have shown that perinatal influences and opiates can affect the development of the HPA-axis and are associated with higher risks of developing neurobehavioral problems. Oxycodone, a semisynthetic putative KOR and partial MOR opioid agonist, is now one of the most frequently abused pain killers during pregnancy. However, limited data are available as to whether and how POE alters the development and function of the HPA-axis, SAM-axis, and neurobehavioral outcomes of the offspring.
We have provided novel evidence that POE indeed is associated with sex-specific changes in the HPA-axis response to stress that persist beyond the neonatal period. POE is associated with an increased ACTH, but not CORT response to CRH, in late adolescent males. This enhanced response is not present in female offspring. Adult OXY female rats, but not OXY male rats, have increased CORT responses to psychological stress, tested by the restraint stress test (RST), but the ACTH response to RST is not altered in either sex. These changes in the HPA-axis response to stress may be partially explained by 1) the increase in the subpopulation of CRH neurons that also contain ERbeta immunoreactivity following POE which then can exaggerate the stimulation of the HPA-axis and 2) the decrease in MR-mRNA expression in the hippocampus which can impair the negative feedback control of the HPA-axis by the limbic system. POE is also associated with cardiovascular changes in response to stress during the classical conditioning paradigm; adolescent male rats exposed to oxycodone in utero have a larger blood pressure increase compared to the control group. Although POE male rats can properly discriminate the stress versus non-stress cues in the paradigm, they seemed to lose the retention of memory when retested during adulthood. When tested for learning and memory with the water maze, however, we did not find any differences between control rats and rats exposed to high dose oxycodone in utero. In contrast, exposure to the lower dose of oxycodone in utero is associated with hyperactivity in adult rats when tested in the open field. Our results make a significant contribution to the literature because they extend our knowledge about the effects of oxycodone on the developing brain and the outcomes in animal models that are actually relevant to a current major public health problem in humans. Furthermore, this will provide a platform for us to further study the underlying mechanisms and interventions that may mitigate these effects.
Figure 8: Summary of the effects of perinatal oxycodone exposure on the HPA-axis, cardiovascular response to stress and behavior/memory
ABBREVIATIONS

ACTH: adrenocorticotropic hormone
BP: blood pressure
CORT: corticosterone
CEA: central nucleus of amygdala
CRH: corticotropin releasing hormone
ER: estrogen receptor
GR: glucocorticoid receptor
HPA-axis: hypothalamic-pituitary-adrenal axis
HR: heart rate
KOR: Kappa opioid receptor
m-RNA: messenger ribonucleic acid
MOR: Mu opioid receptor
MR: mineralocorticoid receptor
OR: opioid receptor
OXY: oxycodone
PFC: prefrontal cortex
POE: perinatal oxycodone exposure
PVN: paraventricular nucleus of the hypothalamus
SAM-axis: sympathetic-adrenal-medullary axis


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B. Book chapters:


**Sithisarn T**: Apnea of Prematurity, in *Advanced Neonatal Care*, Punnahitanonda, S. Active Print Publishing, 2013
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