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From crib to bench: understanding Neonatal Opioid Withdrawal Syndrome (NOWS) using a novel rodent model

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FROM CRIB TO BENCH: UNDERSTANDING NEONATAL OPIOID WITHDRAWAL SYNDROME (NOWS) USING A NOVEL RODENT MODEL

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by
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APPROVAL OF THESIS

We, the faculty supervising the work of Sarah Stevens, affirm that the dissertation, *Crib to Bench: Understanding Neonatal Opioid Withdrawal Syndrome (NOWS) Using A Novel Rodent Model*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I would like to dedicate this body of work to my loving family who have been a constant source of encouragement throughout this journey. To my parents, John and Ruby, thank you for raising me with an appreciation for the importance of education and hard work. You have always pushed me to look beyond my perceived limitations, and assured me that I can achieve anything I put my mind to. This journey has not been easy, but you were both always there to cheer me on and steer me forward through the many ups and downs. It is because of the sacrifices each of you made, that have afforded a better life for me and Elijah, and for that I am grateful. To my brother, Elijah, thank you for your love and support throughout my life and during my academic career. I hope that I can be the same source of support during your journey through law school.

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LIST OF ABBREVIATIONS

NOWS	Neonatal opioid withdrawal syndrome
NAS	Neonatal abstinence syndrome
LC	Locus coeruleus
AC	Adenylyl cyclase
PK	Protein kinases
PTSD	Post-traumatic stress disorder
AD	Alzheimer's disease
PD	Parkinson's disease
MS	Multiple sclerosis
ADHD	Attention-deficit hyperactivity disorder
DTO	Diluted tincture of opium
PND	Postnatal day
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
E	Embryonic day
MOR, μ	Mu opioid receptor
DOR, δ	Delta opioid receptor
KOR, κ	Kappa opioid receptor
HPA	Hypothalamic-pituitary-adrenal
ACTH	Adrenocorticotropin hormone
CORT	Cortisol
LPS	Lipopolysaccharide

LTP	Long-term potentiation
LTD	Long-term depression
GABA	Gamma-Aminobutyric Acid
NMDA	N-methyl-D-aspartate
BDNF	Brain derived neurotrophic factor
PSD-95	Postsynaptic density protein 95
CYP17	Cytochrome P450 17- α -hydroxylase
DHEA	Dehydroepiandrosterone
PMS	Premenstrual syndrome
APC	Activated protein C
G	Gestational Day
S.Q.	Subcutaneous
USV's	Ultrasonic vocalizations
DHEAS	Dehydroepiandrosterone sulfate
ITI	Inter-trial interval
IUGR	Intrauterine growth restriction
GI	Gastrointestinal
CHH	Cabell Huntington Hospital
HC	Head circumference
ANS	Autonomic nervous system
CNS	Central nervous system

ABSTRACT

As the opioid epidemic continues to grow, opioid use among pregnant women is increasing significantly. This has led to a steady rise in the number of infants born with neonatal opioid withdrawal syndrome (NOWS). Although short-term withdrawal symptoms associated with NOWS are well characterized, there are many gaps in our understanding of the short and long-term effects of prenatal opioid exposure. In CHAPTER 1, we describe the clinical presentation, associated neurodevelopmental challenges, and current treatments of NOWS. Our current understanding of the neuropathology of NOWS is limited, and therefore further research is needed. However, current animal models are limited by several confounding factors. In CHAPTER 2, we describe an overview of animal models that have been used to model prenatal opioid exposure and current findings. In CHAPTER 3, we describe a unique rodent species, *Acomys cahirinus*, which may serve as a more translational model for prenatal opioid exposure. In CHAPTER 4, we describe the use of *Acomys cahirinus*, more commonly known as spiny mice, to assess the short-term effects of prenatal morphine exposure. We found that prenatal morphine exposure led to an increase in withdrawal behaviors including wall climbing, face cleaning, jumping, wet dog shakes, tremors, and a decrease in ultrasonic vocalizations in spiny mice pups during the early postnatal period (PND 0 -7). Additionally, physiological changes such as increased body temperature and decreased body weight were observed in morphine exposed offspring. Sex differences were observed in the withdrawal behaviors and physiological changes associated with prenatal morphine exposure. In CHAPTER 5, we describe the long-term effects of prenatal morphine exposure on spatial memory in adolescent and adult spiny mice offspring. A deficit in spatial working and reference memory were observed in morphine exposed offspring. These deficits persisted from adolescence to adulthood in a sex specific

manner. Additionally, we found that morphine exposed offspring had lower body weights compared to saline that persisted from 1.5 – 3.0 months of age, and was more pronounced in female offspring. In CHAPTER 6, we describe a retrospective chart review of infants born at Cabell Huntington Hospital diagnosed with neonatal abstinence syndrome (NAS) from April 2015 – December 2015. Maternal demographics and drug screening information were collected. Infant information was collected during the first 7 days of life including withdrawal symptoms, treatment, and growth parameters. We found significant gender differences in withdrawal behaviors, time to methadone treatment initiation, and total methadone exposure between male and female infants with NAS. The studies included in this thesis validated the use of a unique rodent species, *Acomys cahirinus*, to model prenatal opioid exposure. We described the short-term consequences and withdrawal behavior as well as the long-term consequences on memory in this model. Collectively, these studies demonstrated significant gender differences in withdrawal behaviors, postnatal growth, and memory impairments. Future studies will be focused on understanding the underlying molecular changes in the developing spiny mouse brain following prenatal opioid exposure. We hope to find the molecular basis for our observed gender differences. Additionally, we are hopeful that future studies can ascertain potential therapeutic interventions to prevent NOWS in infants prenatally exposed to opioids.

CHAPTER 1

NEONATAL ABSTINENCE SYNDROME (NAS): NEURODEVELOPMENTAL CHALLENGES, CURRENT TREATMENTS AND FUTURE DIRECTIONS

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ABSTRACT

Opioid dependence is at epidemic levels worldwide and here in the USA it is estimated that over 2.1 million people are suffering from substance use disorders related to prescription opioid pain relievers. With its growth, opioid abuse has significantly extended into the pregnant population, which has allowed for increased risk of adverse pregnancy outcomes such as neonatal abstinence syndrome (NAS). Neonatal abstinence syndrome is a significant cause of morbidity and mortality in term and preterm infants. Neonatal abstinence syndrome has become an epidemic as it was the greatest single reason for neonatal hospital admissions in the United States in 2015. This review summarizes how opioid exposure *in-utero* may affect neurodevelopment and thus neurobehavior in babies born with NAS, with particular emphasis on the Locus coeruleus (LC)-noradrenergic system. We also discuss the currently available pharmacological care for both the opioid abusing mother, as well as the care for babies born with NAS, and provide some narrative on future directions to best help this population of NAS babies as they mature into children and young adults.

Keywords: Addiction; Babies; Brain; Neonate; Morphine

INTRODUCTION

The use and abuse of drugs, alcohol and tobacco contribute significantly to the burden of society. The 2014 National Survey on Drug Use and Health reported that 27 million people aged 12 or older used an illicit drug in the past 30 days. This is approximately 1 in 10 Americans (10 percent) (K. A. Mack, Zhang, Paulozzi, & Jones, 2015). The increase in illicit drug use continues to be driven by marijuana (22.2 million illicit users) and the nonmedical use of prescription drugs such as pain relievers (4.3 million illicit users), with the 18 to 25-year-olds being the largest group of illicit users (Campbell et al., 2010). Compared to the illicit use of prescription opioid pain relievers, heroin use has increased in the population; it is estimated that heroin use in 2014 among people aged 12 or older was higher than that estimated between 2002 and 2013. Individuals prescribed opioids for pain frequently become tolerant to the analgesic properties of these drugs such that, if not controlled, can often lead to the development of addiction. Following the completion of the prescribed pain therapy regimen, patients may seek to satisfy their addiction elsewhere, such as with the purchase of illicit prescription opioids and they may subsequently turn to heroin as an easier, cheaper alternative to achieve the desired reward. With current data suggesting that the highest incidence of opioid abuse is among the Medicaid population, this form of substance abuse is widely associated with lower-income individuals and those who are already predisposed to a higher risk of physical and mental health illnesses (K. A. Mack et al., 2015).

The most frequently prescribed opioids among women in both the Medicaid and privately insured groups are hydrocodone, codeine and oxycodone. Long-term opioid use amongst the female population of Medicaid enrollees in 2005 treated for non-cancer pain, ranged from 62% among those aged 19 to 44 years to 98% among those aged 45 to 65-year-old (Campbell et al.,

2010). Thus, it is evident that more interventions are needed to better manage opioid treatment and perhaps improvements are needed to help educate females of childbearing age, as it is important to understand the effect prenatal opioid use has on the fetus and thus the short and long-term effects on a child's development. Opioid exposure during childbearing ages (15 to 44 years) suggests increased risk of adverse pregnancy outcomes, such as Neonatal abstinence syndrome (NAS) and long-term birth defects. Neonatal abstinence syndrome has become an epidemic, as evidence has shown that it was the single greatest cause for neonatal hospitalizations in the US in 2015 (Tolia et al., 2015). Between 2000 and 2012, there has been a five-fold increase in the incidence of NAS, from 1.2 to 5.8 live births per 1,000 diagnosed respectively. During this 12-year period of time it was seen that infants born with NAS occupied, on average 16.9 days' hospital stay postpartum, relative to the 2 days seen by most non-NAS infants (Tolia et al., 2015).

Opioids are most commonly seen in the precipitation of NAS and work through the G protein-coupled receptors (mu, kappa and delta opioid receptors) located throughout the central and peripheral nervous systems, as well as the gastrointestinal system (Feng et al., 2012). Notably, neonates are born with mu opioid receptors similar to those of an adult, while kappa and delta opioid receptors are still developing in the neonatal brain (Barr, McPhie-Lalmansingh, Perez, & Riley, 2011). Neonates chronically exposed to opioids and thus activation of these receptors while *in-utero,* will experience symptoms of NAS with the abrupt discontinuation of these mechanisms after birth. This leads to increased activation of adenylyl cyclase (AC) activity, ionic imbalances and increased neurotransmitter activity through enzymatic cascades. Presentation of NAS symptoms are suggestive of such imbalances and are subsequently managed through medicinal opioid therapies, to first achieve homeostasis and then to slowly wean the

infant off the potential opioid substance either in question or detected via analytical laboratory tests.

Neonatal abstinence syndrome is the manifestation of symptoms presented by newborns shortly after birth due to the abrupt discontinuation of trans-placental exposure to licit or illicit substances throughout pregnancy. Neonatal abstinence syndrome presents as a variety of symptoms that include neurological: tremors, irritability, wakefulness, exaggerated Moro reflex, seizures, sneezing and yawning; gastrointestinal: poor feeding, vomiting, diarrhea, dehydration, poor weight gain; and autonomic dysfunction: fever, temperature instability, sweating, nasal stuffiness, and mottling (Kocherlakota, 2014). Based on a national survey taken in 2006, the most widely (65%) used method to diagnose NAS and measure base lines to initiate treatment in the US is the Finnegan Scoring System (Finnegan, Connaughton, Kron, & Emich, 1975). The Finnegan scoring system was developed in 1975 as a clinical, investigative tool to quantify the severity of NAS in full-term babies that presented with symptoms. The Finnegan scoring system assesses the passively addictive infant in a comprehensive and objective manner to help provide an accurate evaluation of the clinical state of the NAS infant undergoing withdrawal. This scoring system can be helpful for initiating, monitoring and terminating treatment in neonates. This scoring system has also allowed for the development of set, uniform criteria for the assessment and treatment of the NAS infant to the addicted mother following discharge. Diagnoses and treatment of clinical symptoms has become substantially more complex as the variety and type of drugs being abused *in-utero* has increased. Additionally, the duration of prenatal drug exposure can significantly impact the diagnose of NAS, and thus its impact on neurodevelopment (Kocherlakota, 2014). However, before we discuss changes in neurodevelopment, it is important to state that the symptoms associated with polysubstance-

mediated NAS can correlate and are generally more severe than when compared to the abuse of a single drug substance such as hydrocodone. The primary focus of this article is to highlight the potential developmental challenges that could present in infants diagnosed and treated for NAS following prenatal opioid exposure.

NEURODEVELOPMENTAL CHALLENGES OF NAS

Currently, the neuropathology behind opioid-mediated NAS is unknown and, therefore, warrants further research to improve our understanding of the mechanisms responsible for opioid withdrawal in neonates – this could positively impact the clinical outcomes (short and long-term) of all babies born with NAS.

The physiochemical properties of opioids (i.e. lipophilicity) allows for easy transfer across the placenta to the fetus, and this transmission of opioids increases as gestation increases (Nanovskaya, Nekhayeva, Hankins, & Ahmed, 2008). To improve our understanding of the mechanism responsible for opioid withdrawal in neonates, a better understanding of how opioids metabolize in the placenta and fetus is needed. Opioid withdrawal is a complex phenomenon in adults and is further complicated in neonates due to immature neurological development and variations between fetal exposure to opioids (i.e. pharmacokinetics) mediated by the placenta.

Improving our understanding of the *in-utero* metabolism and effects of opioids will allow us to predict how sustained exposure may affect the development of the neonate and then develop potential treatment strategies (i.e. agents and treatment protocols) that may improve clinical outcomes of these babies.

Opioid Receptor Signaling

Using opioid withdrawal data obtained from research conducted in adults, it is possible to predict the impact of opioid exposure may have on the developing brain. For example, opioid receptors-mu, kappa and delta are expressed throughout the CNS in both adults and neonates, and therefore, similar G-protein-driven signaling could be predictable. During opioid withdrawal, stress, growth factors and neurotransmitters can cause a cellular super-activation of adenylyl cyclase (AC)/cAMP signaling which then can result in increased activity of protein kinases (PK) (i.e. mitogen activated PK) and increased expression of transcription factors (i.e. CREB) (Christie, 2008). If not regulated, the pathological consequences of CREB activation could result in either increased or decreased levels of neurotransmitters (i.e. norepinephrine and glutamate) that might be responsible for some of the symptoms that characterize NAS. For example, during opioid withdrawal, supraphysiological levels of norepinephrine can cause hyperthermia and tremors in babies with NAS (Little, Price, Hinton, & Kuhn, 1996). In contrast, decreased levels of serotonin in the CNS can be responsible for sleep deprivation in babies with NAS (Lunden & Kirby, 2013).

Locus coeruleus (LC)-noradrenergic system

A focus of this article is to highlight regions of the brain that are affected by sustained opioid exposure in the developing fetus. A key region of the brain involved in opioid addiction and subsequent withdrawal is the locus coeruleus (LC) of the pons (Van Bockstaele, Reyes, & Valentino, 2010). The LC is involved with the physiological response to stress and panic and as the principle site for the synthesis of noradrenaline it known as the LC-noradrenergic system. The LC is composed of medium-sized neurons and has high concentrations of neuromelanin

formed by the polymerization of noradrenaline. The projections of the LC are vast and potential damage to the nucleus of the pons during development could be devastating. For example, the LC innervates the spinal cord, brain stem, cerebellum, hypothalamus, thalamus, tectum, amygdala, ventral tegmental area and the cortex. These projections from the LC are noradrenergic and, therefore, generally excitatory and thus mediate arousal and prime the brain to be activated and ready to respond to stimuli. In addition to outputs, the LC also receives inputs from numerous regions of the brain. For example, the medial prefrontal cortex, nucleus *paragigantocellularis lateralis* (located in the ventrolateral medulla) and the nucleus *praepositus hypoglossi* all provide inputs to the LC. Therefore, due to its broad function and roles, damage to and diminished development of the LC-noradrenergic system in the brain of the fetuses exposed to opioids could be responsible for changes in neurodevelopment and behavior in NAS babies later in life.

Noradrenergic neurons were once thought to be exclusively present in the LC; however, noradrenergic neurons are also found scattered throughout the lateral tegmental area of the pons and medulla. Due to the extensive projections of the midbrain noradrenergic neurons from the LC, it is widely thought that the LC has functional roles in all forebrain activities, including perception, cognition and memory formation (Sara, 2009). The function of noradrenergic neurons from the LC is tightly regulated and thus correlated with the state of arousal (Izumi & Zorumski, 1999). In addition to the activation of noradrenergic neurons to stimuli, many studies have shown that noradrenaline promotes changes in synaptic function by 'gating' the induction of long-term synaptic plasticity of glutamatergic synapses (Alreja & Aghajanian, 1993; Salgado, Treviño, & Atzori, 2016). Therefore, with its repertoire of effects, the hypothesis that the LC-noradrenergic system is critical in coordinating the activity of cortical and subcortical circuits for

the integration of sensory activity and working memory could mean that babies with NAS could have changes in cognitive functions such as perception, attention, learning and memory.

In opioid withdrawal, the LC-noradrenergic system is hyperactive partly due to increased glutamate transmission provided by input from neurons in the nucleus *paragigantocellularis lateralis* and by increased hyperactivity in regions of the brain that are innervated by these neurons. Noradrenergic neurons of the LC are inhibited by acute exposure to exogenous opioids, a mechanism mediated by activation of inward-rectifying K+ currents through coupling with the Gi/Go family of G-proteins (Andrade, Vandermaelen, & Aghajanian, 1983). Following chronic opioid exposure, LC neurons develop cellular tolerance and dependence where they gradually recover from the acute inhibitory effects of opioid, and when in withdrawal to opioids, LC neurons exhibit hyperexcitability (Akaoka & Aston-Jones, 1991; Rasmussen & Aghajanian, 1989). The mechanisms responsible for this neuronal hyperexcitability can be explained by the upregulation of cAMP in the LC neurons and increased glutamatergic transmission from the nucleus *paragigantocellularis lateralis* during opioid withdrawal (J. A. Ross, McGonigle, & Bockstaele, 2015; Tokuyama, Zhu, Wakabayashi, Feng, & Ho, 1998).

Functions of the noradrenergic neurons projecting from the LC include modulation of attention, sleep-wake states and mood. Increased firing of the LC neurons increases the awake state that often presents in babies with NAS and if not controlled, can eventually lead to degeneration and contribute to cognitive decline (Y. Kim et al., 2015; Yan Zhu, Fenik, Zhan, Xin, & Veasey, 2015). Chronic patterns of sleep deprivation can induce long-term changes in the brains adenosine and noradrenaline receptors which may, underlie the long-lasting neurocognitive impairment observed in chronic sleep restriction (Tobaldini et al., 2017). Also, chronic patterns of sleep deprivation can induce changes in cholesterol metabolism, the integrity

of the blood-brain barrier, pain experiences, immune responses, neurogenesis, mood, food intake, motivation, weight, sexual arousal and blood pressure as well as impair learning and memory formation and retrieval (Wahlsten & Sarman, 2013). Therefore, the impact of sleep disturbances in babies born with NAS could have on the physiological and neurobehavioral levels are significant, and therefore, could potentially echo into their childhood and even adulthood. Dysregulation of the LC has been proposed to contribute to behavioral disorders such as insomnia and narcolepsy; neuropsychiatric conditions such as post-traumatic stress disorder (PTSD), depression and anxiety; and neurogenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS). Additionally, attention-deficit hyperactivity disorder (ADHD) has been shown to be more prevalent in children born to opioid-addicted mothers when given buprenorphine (Wahlsten & Sarman, 2013). In a recent study, NAS (Finnegan) scores were strongly associated with poor and deteriorating academic performance (Oei et al., 2017). Plausible reasons for this correlation are vast and may encompass parental education, socio-economic hardships, quality of the school and other environmental factors. Damage to the LC neurons and other brain nuclei inflicted *in-utero* may also contribute to impaired academic performance seen in children born with NAS; however, the degree of the impact remains unknown. Additionally, increased inflammation in LC-noradrenergic neurons can cause the functions of NA to switch from neurotrophic and neuroprotective to a more pro-inflammatory function due to the increased activation of non-neuronal cells such as macrophages and glia (Feinstein, Kalinin, & Braun, 2016). Chronically, a pro-inflammatory state can cause LC neuronal cell loss that can lead to neurogenerative disease's such as AD and MS (Berne-Fromell, Fromell, Lundkvist, & Lundkvist, 1987; Trillo et al., 2013).

During opioid exposure, the levels of endogenous opioid peptides (i.e. encephalin) decrease and, therefore, provide a diminished role in inhibiting noradrenergic neurons (Elisabeth J. Van Bockstaele, Menko, & Drolet, 2001). Using rodent models of chronic morphine withdrawal, prodynorphin and FK506 binding protein 5 were two genes found to be strongly regulated by chronic morphine exposure withdrawal in the LC. They were and associated with regulating withdrawal-associated behaviors, and when inhibited, profoundly affected withdrawal responses (McClung, Nestler, & Zachariou, 2005). Additionally, prodynorphin is the building block of endorphins that are important in the experience of pain, emotional bonding, learning and memory (Chavkin, Shoemaker, McGinty, Bayon, & Bloom, 1985). Therefore, it may be possible that NAS babies have alternations in their ability to experience pain, form bonds and generate memories from significant changes in the expression and function of prodynorphin. The role of the LC-noradrenergic system is important to everyday activities and emotions. The LC-noradrenergic neurons project to various regions of the brain that are key in regulating mood, including the hypothalamus, thalamus, amygdala and prefrontal cortex (Jacobson, 2014; Navarra, Clark, Gargiulo, & Waterhouse, 2017). However, in a baby born with NAS the LC-noradrenergic system can fail to be correctly regulated, resulting in significant hyperactivity of the various functions of the LC-noradrenergic system. Current treatments used to help control withdrawal symptoms are associated with opioid replacement therapy and the noradrenergic system. However, the long-term effects of a dysregulated LC-noradrenergic system remain unknown and warrant further studies with focus on prolonged treatment options, both *in-utero* (i.e. neuroprotectants) and postnatally, to reduce the risk on long-term, lifelong changes in neurobehavior.

CURRENT TREATMENTS

Opioid withdrawal during pregnancy: Methadone and Buprenorphine

FDA approved treatment options available to opioid-dependent women prior to and during pregnancy are currently limited to Dolophine® (methadone), Subutex® (Buprenorphine), Suboxone® (Buprenorphine plus Naloxone) and Vivitrol® (Naltrexone). Methadone was first introduced as a replacement treatment of opioid addiction in 1964 (National Consensus Development Panel on Effective Medical Treatment of Opioid Addiction 1998). Methadone use during pregnancy was at first believed to not cause withdrawal in neonates, but this was later found to be inaccurate (Reddy, Harper, & Stern, 1971). However, methadone, a synthetic complete mu-opioid receptor agonist, has become the standard of care for pregnant women with opioid addiction. Methadone maintenance treatment during pregnancy can improve obstetric care, decrease illicit drug use and improve fetal outcomes (Kandall, Doberczak, Jantunen, & Stein, 1999). However, pharmacokinetics studies on methadone during pregnancy have found that higher doses are required to elicit a pharmacological effect (Jarvis, Wu-Pong, Kniseley, & Schnoll, 1999; Pond, Kreek, Tong, Raghunath, & Benowitz, 1985).

Buprenorphine, a semi-synthetic partial mu-opioid receptor agonist and competitive kappa-opioid receptor antagonist, has comparable efficacy to methadone and has fast become an alternative to methadone for the treatment of opioid addiction during pregnancy. Buprenorphine was approved as an alternative to methadone for opioid addiction in Europe and the USA in 1996 and 2002, respectively. Buprenorphine, being a partial mu-opioid receptor agonist, may also contribute to the occurrence of NAS neonates exposed *in-utero* (Jones et al., 2010). One study showed that infants born to mothers who took buprenorphine throughout pregnancy required less morphine overall (1.1 mg vs 10.4 mg, respectively), had significantly shorter hospital stays (10.0

vs 17.5 days, respectively) and had shorter treatment durations for NAS (4.1 vs 9.9 days, respectively) when compared to infants of mothers who took methadone throughout pregnancy. In a recent retrospective study comparing methadone and buprenorphine use in pregnancy from 2009 to 2012, buprenorphine use increased from 10.1% to 33.2%, but overall, methadone continued to be more prevalent (76.9%) compared to buprenorphine (23.1%) when used to treat pregnant women for opioid addiction (Krans et al., 2015). Additionally, a 2014 meta-analysis study comparing methadone versus buprenorphine in the treatment of pregnant women addicted to opioids found that treatment with buprenorphine was associated with shorter hospital stays, higher mean gestational age and greater mean birth weight, length and head circumference (Brogly, Saia, Walley, Du, & Sebastiani, 2014). Nevertheless, despite the observed advantages of administering buprenorphine compared to methadone in pregnancy, disparities still exist, and there may be a need to improve the availability of buprenorphine for the treatment of pregnant women addicted to opioids.

Opioid Withdrawal in Neonates: Morphine, Methadone and Buprenorphine

Alcohol-free morphine sulfate and morphine hydrochloride solutions have been shown to be effective treatments for NAS infants (Osborn, Jeffery, & Cole, 2010). Diluted tincture of opium (DTO) has also been used to treat NAS, however, this drug is more toxic to neonates due to its alcoholic content. Therefore, the use of DTO has been greatly reduced in recent years in favor of morphine, methadone and buprenorphine. Morphine is one of the most commonly used and preferred agents for treating NAS-afflicted newborns due to its predictable pharmacokinetic profile, half-life and pharmacologic effects. Morphine mitigates and decreases the rates of seizures, diarrhea, agitation and poor feedings. However, it must be provided every 3 to 4 hours

due to its short half-life. Nevertheless, it is more easily titrated versus methadone, the half-life of which can vary between 18 and 70 hours between patients (Kocherlakota, 2014). In a 2005 retrospective study, it was shown that only a little difference existed between the lengths of stay between infants treated with methadone versus morphine. In this study, length of stay was also found to be directly related to the overall amount of morphine or methadone given, which correlated directly with the severity of the withdrawal symptoms (Sarkar & Donn, 2006). In a more recent study comparing methadone to morphine for the treatment of NAS, methadone significantly decreased the length of opioid withdrawal treatment of babies compared to morphine treatment alone (14 vs. 21 days, respectively) (Beaulieu, 2013). To compare weaning versus non-weaning protocols, a multicenter cohort study published in 2014 found that infants treated with opioids according to a weaning protocol had significantly shorter durations of hospital stay when treated with opioids then compared to infants who were not treated according to any weaning protocol (Agthe et al., 2009). Additionally, this study identified that the levels of stringency and adherence to the protocols played a more impactful role in clinical outcomes versus less-stringent and variable protocols. Incidentally, when comparing the levels of stringency and adherence to the protocols, no clinically significant difference between methadone and morphine weaning protocols was found, but a clinically significant increase in the length of stay was found when infants were treated with phenobarbital in addition to morphine (Agthe et al., 2009).

Phenobarbital, Clonidine and Gabapentin

In addition to using methadone and morphine to treat withdrawal, additional agents can also be used to treat the various array of symptoms associated with NAS. For example, the

sedative and anti-seizure effects of phenobarbital and chlorpromazine can benefit neonates through the relief of irritability and trembling and by reducing the occurrence of seizures (Surran et al., 2013). A Cochrane systematic review conducted in 2010 summarized that use of phenobarbital to ameliorate symptoms associated with NAS resulted in a significant reduction in the duration of daily supportive care needed versus supportive care alone (Surran et al., 2013). Phenobarbital was also concluded to be preferable to diazepam, which had a higher risk of treatment failure. Chlorpromazine and clonidine were also compared to phenobarbital, with no statistical difference found in efficacy in regards to the rate of treatment failure, seizure occurrence, or overall mortality. Phenobarbital is one of the preferred adjunctive agents for neonates of mothers that abused multiple substances though out pregnancy and hence in many cases, phenobarbital is added to morphine or methadone therapies (Sarkar & Donn, 2006; Surran et al., 2013). Clonidine is a centrally acting α_2 adrenergic receptor agonist used as adjunct therapy in opioid withdrawal in children and adolescents. Clonidine-mediated stimulation of presynaptic adrenergic receptors causes the inhibition of CNS sympathetic outflow and the reduction of norepinephrine release. In relation to withdrawal, clonidine reduces autonomic over-activity such as tachycardia, hypertension, diaphoresis and diarrhea. Clonidine has been shown to reduce the overall length of hospitalization and duration of treatment. Clonidine also has fewer risks of respiratory depression or sedation versus opioid analgesics however, hypotension can occur (Evoy, Morrison, & Saklad, 2017). In relation to NAS, oral clonidine, when used in a randomized, double-blind trial as an adjunct to standard opioid therapy for detoxification, reduced the duration of pharmacotherapy for NAS; however, the long-term impact on neonate development and recovery remains unknown (Loudin et al., 2017). Phenobarbital and clonidine

are currently used as adjunct therapy with opioids to reduce the severity of neonatal withdrawal symptoms (Langenfeld et al., 2005; Surran et al., 2013).

Only recently has nonprescription abuse of gabapentin been found to be increasing among opioid abusers (Brown, Hayes, & Thornton, 2015). Therefore, babies born to mothers who have potentially abused both opioids and gabapentin have an observed atypical withdrawal syndrome. In a recent study, it was shown that these NAS babies that presented with this atypical withdrawal syndrome (i.e. tongue thrusting, wandering eyes, back arching and continuous extremity movements) in response to opioid and gabapentin exposure can be successfully treated with gabapentin if these "gabapentin-babies" failed to wean in response to escalating doses of methadone alone (Hall et al., 2014).

Nonpharmacological treatment

Despite the data supporting pharmacologic therapy of NAS-afflicted neonates, nonpharmacological care is still the mainstay of treatment. Care such as swaddling, gentle handling, demand feeding and conscientious maintenance of a quiet environment are key to minimizing reactionary symptoms of NAS such as irritability, excessively high-pitched crying, tremors and Moro-reflex hyperactivity. Nonpharmacological therapy can successfully manage milder cases of NAS without resorting to pharmacologic interventions. However, no study to date has compared specific methods of nonpharmacological care. It has also been shown that permitting the mothers to room with, swaddle and participate in the treatment of their infant can significantly reduce the need for pharmacological intervention (Pritham, 2013; Welle-Strand et al., 2013). Additionally, breastfeeding may reduce the severity of NAS symptoms and the infant's requirements for morphine or methadone (Welle-Strand et al., 2013). This may be due to the comforting unbreakable bond created between mother and child, the quietness of the

environment, the drug content of the mother's breastmilk or any combination of these or other factors (Hodgson & Abrahams, 2012). Overall, it has been shown that infants with mild cases of NAS do not always require pharmacological care when the mother is present and participating in their nonpharmacological care.

Overall, there is no individual, standardized treatment protocol that is first-line when treating NAS. There are various treatment algorithms that involve varying pharmacological agents that depend on various factors such as availability and hospital or clinic experience. Although certain agents have been shown to be superior to other treatment options, variability remains between patient populations. Additionally, patient-specific factors such as infant gender, gestational age, pharmacogenomics and the degree and duration of exposure to any of several substances *in-utero* further complicate matters.

CONCLUSION

Neonatal abstinence syndrome is a national epidemic and, thus, with the increased financial burden of treating newborns across the United States, it is pivotal to understand the short-and long-term impacts NAS will have on these children. To date, there have been only a few studies that have documented the effects on fetal growth and infant behavior (Coyle et al., 2012; Kivistö et al., 2014; Velez, Jansson, Schroeder, & Williams, 2009). Currently, though, there is no consensus as to the effects of prenatal opioid exposure may have on cognition, language and learning. With the current rate of babies being born with NAS it is important to not only continue to treat the withdrawal symptoms, but to also begin to understand the short-term (1-5 years) and long-term (5-16 years) impact of NAS. To help us predict the possible short- and long-term problems we need to take a multipronged approach.

The major branches of this multipronged approach need to include an improved understanding of the functional outcomes, as well as networking within the regions of the brain and how it may impact neurobehavioral traits as these children develop and mature into young adults. It is also important to understand the possible changes in genes that may occur in babies born with NAS. A better understanding of pharmacogenomics might be more important in this cohort of the population, as having *in-utero* exposure to opioids may affect may have affected the expression of proteins used to metabolize drugs and the bioavailability of various drugs. Thus, NAS babies might present with adverse effects and complications not commonly observed in neonates with a specific class of drugs. Lastly, it is important to improve the current systems used to record the birth, diagnosis, treatment protocols and outcomes before, during and after treatment as well as before discharge for withdrawal in babies born with NAS. To help improve this, the systems used to track the wellbeing of children would need to be updated with a specific focus on NAS babies, as this population might be subject to greater challenges than non-NAS babies. Everything about NAS is challenging, but our overarching goal should be to provide equal opportunities to all children, regardless of background (i.e. NAS). If we do not record and share data about children born with NAS, we may encounter, perhaps, greater challenges that come with a viscous cycle rooted in difficulties in learning, socio-economic hardships and transgenerational behaviors of substance abuse. Continued and further efforts are needed to build a public database containing information about current clinical and basic research and clinical trials. Also, a separate securely-protected database containing information about children born with NAS could be built and accessed by healthcare providers, school administrators, teachers, caregivers, child protective services, fostering and adoption services and state and local

departments of health, with the goal being to provide these children with the best environment to allow them to mature into productive and impactful members of society.

Conflict of Interest

The authors declare that they have no competing interests.

CHAPTER 2

PRECLINICAL MODELS OF PRENATAL OPIOID EXPOSURE

INTRODUCTION

As the number of infants exposed to opioids continues to grow, we still do not fully understand the repercussions of prenatal drug exposure and the associated withdrawal symptoms. Factors such as the type of opioid, dosages, frequency, duration, and timing of exposure can impact the development and severity of NOWS. Other factors including polysubstance abuse, gestational age, genetic vulnerability, and even gender can potentially influence the manifestation of this withdrawal syndrome.

Although the short-term withdrawal symptoms associated with NOWS are well characterized (Kocherlakota, 2014), we are still unable to predict if an infant will develop NOWS following prenatal opioid exposure and how severe their symptoms may be. Additionally, there are currently no therapeutics available to prevent the impact of prenatal opioid exposure and the effects it may have on the developing fetus. Similarly, the long-term consequences of NOWS are currently unknown. Currently, there are a limited number of longitudinal studies devoted to investigating the long-term consequences of NOWS, and even fewer that extend past childhood. Longitudinal studies of infants diagnosed with NOWS are difficult as confounding variables such as socioeconomic status, environmental stability, poor parenting, healthcare access and follow up have been shown do impact development (Hatzis, Dawe, Harnett, & Barlow, 2017; Kim et al., 2018; LaGasse, Seifer, & Lester, 1999; Messinger et al., 2004). There is still much to be understood regarding the consequences of maternal substance abuse and prenatal opioid exposure.

Over the past several decades researchers have worked to develop preclinical models of prenatal opioid exposure to further our understanding of its behavioral, physiological, and neural effects on offspring (Byrnes & Vassoler, 2017). However, there are a number of inconsistencies between preclinical models that influence offspring outcomes between different studies. These differences limit the generalization and translatability of preclinical findings (Grecco & Atwood, 2020)

COMMONLY USED ANIMAL MODELS

Over the past five decades, a variety of animals have been used to study the effects of prenatal opioid exposure and opioid withdrawal on the developing fetus. To date, rats have been the most commonly used animal in these studies. However, mice, guinea pigs, and chickens have also been utilized to model prenatal opioid exposure (Byrnes & Vassoler, 2017; Richardson, Yohay, Gauda, & McLemore, 2006). Comparisons between animal models and human studies of prenatal morphine exposure are limited due to the differential timelines of in utero development observed between species. For example, the short gestational period of 18-22 days observed in rats and mice allows for limited in utero development such that at birth, these rodents are comparable to a late second trimester to early third trimester human fetus (Fig. 1.1) (Ross, Graham, Money, & Stanwood, 2014). Therefore, third trimester neurodevelopmental events that occur in humans take place during the early postnatal days in rodents, with rats and mice being equivalent to a human fetus at birth at postnatal day (PND) 7 (Dobbing & Sands, 1979; Hofman, 1983; Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013).

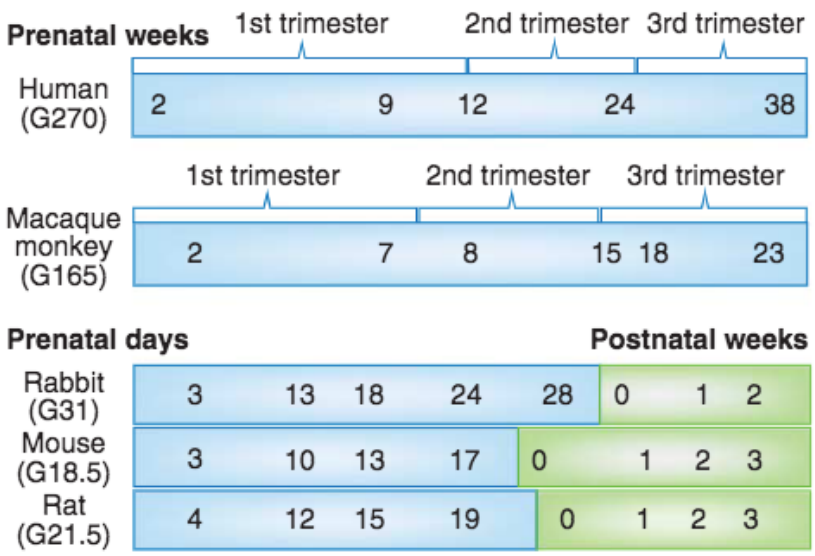

Fig. 2.1 Major neurodevelopmental events across species.
Schematic diagram that aligns human brain development with several animal models (monkey, rabbit, rat, and mouse) often used in studies of fetal drug exposure. Note in particular that the rodent equivalent of third trimester fetal development occurs postnatally. (Ross et al., 2014) Reprinted by permission from Springer Nature: Springer Nature. Neuropsychopharmacology. Developmental Consequences of Fetal Exposure to Drugs: What We Know and What We Still Must Learn. Ross et al., © 2014.

The guinea pig has also been used to model prenatal opioid exposure (Nettleton, Wallisch, & Olsen, 2008; Wallisch, Subban, Nettleton, & Olsen, 2010). Mostly, due their haemomonochorial placentas which are similar to that of the human placenta (Carter et al., 2006; Mess, Zaki, Kadyrov, Korr, & Kaufmann, 2007; Mitchell & Taggart, 2009; J. L. Morrison et al., 2018). In addition, morphine metabolism is similar among guinea pigs and humans. Guinea pigs are capable of producing both morphine metabolites, murophine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Lawrence, Michalkiewicz, Morley, Mackinnon, & Billington, 1992; Murphey & Olsen, 1993). Guinea pigs have a significantly longer gestational period (on average 65 days) compared to other rodents such as the rat and mouse (Dobbing & Sands, 1970).

This allows for a greater degree of in utero development and consequently, smaller litter sizes (app. 2-5 pups/litter). Their increased development at birth and small litter sizes make them a more ideal animal to model in utero morphine exposure compared to rats or mice. However, the growth rate and development of the brain occurs much earlier in gestation in guinea pigs compared to humans (Fig. 1.2) (Dobbing & Sands, 1979). Therefore, in utero brain development still limits the translatability of this species as a model for prenatal opioid exposure for the purposes of understanding neurological and behavioral consequences.

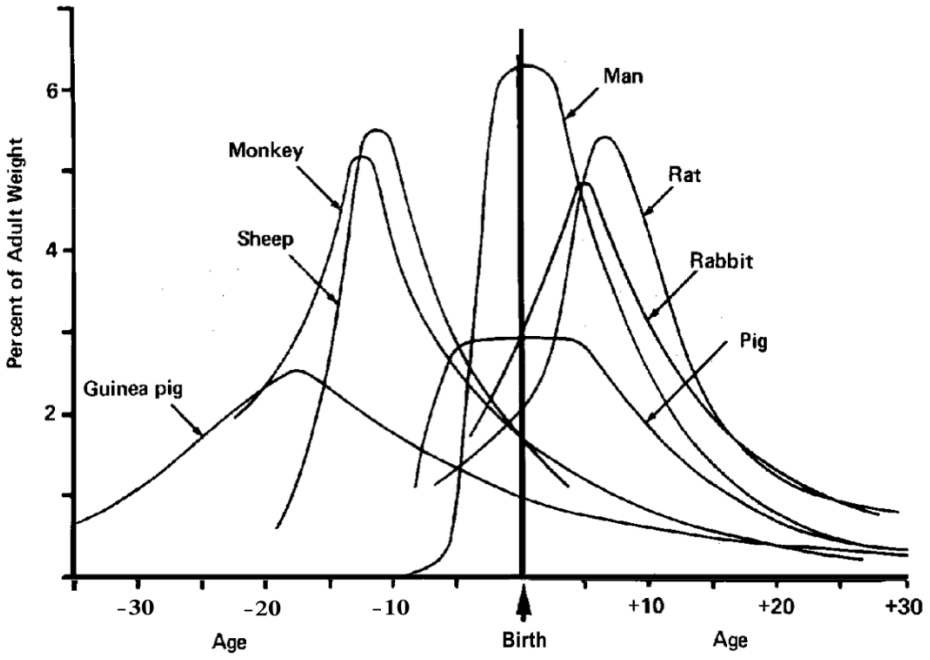

Fig. 2.2 The brain growth spurts of 7 mammalian species expressed at first-order velocity curves of the increase in weight with age.
The units of time for each species are as follows: guinea pig: days; rhesus monkey: 4 days; sheep: 5 days; pig: weeks; man: months; rabbit: 2 days; rat: days. Rates are expressed as weight gain as a percentage of adult weight for each unit of time. (Dobbing & Sands, 1979)

Lastly, several studies have used chickens as a model for prenatal opioid exposure (Che et al., 2005; He, Bao, Li, & Sui, 2010; Jiang, He, Wang, & Sui, 2011; Schrott & Sparber, 2004; Wang, Yao, Nie, & He, 2017; Wang, Yao, Li, Nie, & He, 2017). Previous studies have suggested the chicken to be an ideal model for embryogenesis and developmental studies (Davey & Tickle, 2007). The use of chickens eliminates the possible effects of maternal toxicity in a model of prenatal opioid exposure and allows for a greater understanding of the direct effects opioids have on the developing fetus without additional confounders (Hamburger & Hamilton, 1992; Newby-Schmidt & Norton, 1981). Although chick embryos can easily be manipulated, it's important to note that administration of opioids directly to the fetus bypasses transplacental exposure that occurs during human prenatal opioid exposure.

OPIOID EXPOSURE

Length, method, and timing of opioid exposure are all important factors to account for in preclinical models of prenatal opioid exposure, and are major sources of variability between models. Animal models of prenatal opioid exposure can be divided into two treatment paradigms: *acute* and *chronic* exposure. Acute opioid exposure is considered to be the administration of an opioid given once or multiple times over the course of 1 to 2 days. In contrast, chronic exposure is defined as the repeated administration of an opioid for a minimum of 5 days (Richardson et al., 2006).

Most studies utilize injections (subcutaneous, intraperitoneal, or intravenous) for drug administration (Sithisarn et al., 2017; Slamberova, Bar, & Vathy, 2003; Wu et al., 2014). The use of injections allows for intermittent drug exposure mimicking human drug use. However, the method and frequency of injections can induce stress and anxiety in dams which may impact maternal behavior. Continuous drug exposure can be achieved by using osmotic mini pumps or

subcutaneous pellets, and is commonly used among animal studies (Kunko et al., 1996; Tempel, Yang, & Basheer, 1995; Vestal-Laborde, Eschenroeder, Bigbee, Robinson, & Sato-Bigbee, 2014). However, the use of mini pumps and pellets prevents the ability to account for weight gain adjustments needed for proper dosing throughout pregnancy. Additionally, continuous drug exposure does not accurately model most patterns of drug use in humans. Opioids have also been added to drinking water allowing for oral administration during pregnancy (Alipio et al., 2020; Boggess et al., 2020; Haydari, Miladi-Gorji, Mokhtari, & Safari, 2014). Although this is most likely the least stressful method and more accurately mimics human drug use, it is difficult to determine the amount and dosage each animal is exposed to thus introducing variability among experimental subjects.

The duration of opioid exposure greatly differs among animal models of prenatal opioid exposure. Most studies begin opioid exposure during gestation, often times at mid-gestation in order to coincide with the timing of opioid receptor development in the fetus which begins in the peripheral tissues around embryonic day (E) 9.5, and in neural tissue around E 12.5 (Devarapalli et al., 2015; Hung et al., 2013; Khachaturian, Alessi, Munfakh, & Watson, 1983; Loh, Rius, Elkabes, Bem, & Coscia, 1991; Slamberova, Riley, & Vathy, 2005; Zhu, Hsu, & Pintar, 1998). In contrast, other studies initiate drug exposure prior to conception and continue throughout gestation (Davis, Franklin, Johnson, & Schrott, 2010; Kongstorp et al., 2020; Wallin, Bowen, Roberge, Richardson, & Brummelte, 2019; Yang et al., 2003). This treatment paradigm likely models opioid use during pregnancy most accurately, as most women do not initiate opioid use during pregnancy.

Lastly, in some studies, opioids are administered to pups during the early postnatal period to mimic third trimester exposure. One method of postnatal exposure is direct injections to pups

beginning at birth (Maeda et al., 2002; McPhie & Barr, 2009; Robinson, Jones, Brynildsen, Ehrlich, & Blendy, 2019; Tempel & Espinoza, 1992; Tempel, Habas, Paredes, & Barr, 1988). This method of exposure likely induces significant stress in pups due to repeated handling and painful injections. Direct injections given to neonatal pups adds the confounding variable of increased stress which may have a significant impact on offspring. Several studies administer opioids to pups through the dam's breastmilk (Jantzie et al., 2020; Kongstorp et al., 2020; Kunko et al., 1996; Tempel et al., 1988; Timár et al., 2010). Although passive drug exposure through maternal milk removes the stress of direct injections, this likely results in varying amounts of drug being passed on to pups within a litter. Additionally, this method of exposure relies on the assumption that opioids are transferred from mother to pup via breastmilk at pharmacologically relevant levels. However, it is difficult to assess the opioid concentrations in dams and offspring during the early postnatal period. Studies that have assessed opioid levels have reported relatively low opioid concentrations in pups that were postnatally exposed to opioids via breastmilk (Jantzie et al., 2020; Kongstorp et al., 2020; Kunko et al., 1996). Additionally, postnatal treatments remove the transplacental exposure that takes place during human pregnancy therefore limiting the translatability of these methods.

CONSEQUENCES OF PRENATAL OPIOID EXPOSURE IN ANIMAL MODELS

Opioid Withdrawal in Rodent Neonates

There are two experimental study designs to assess the effects of opioid exposure and withdrawal: *spontaneous* and *precipitated* opioid withdrawal. *Spontaneous* opioid withdrawal occurs after an abrupt discontinuation of opioid exposure. Precipitated opiate withdrawal utilizes an opioid receptor antagonist such as naloxone or naltrexone to bring about withdrawal. Both

spontaneous and precipitated withdrawal result in the negative withdrawal symptoms, however the onset of these symptoms differs between these two models with precipitated opioid withdrawal occurring much quicker than spontaneous withdrawal (Richardson et al., 2006). It is important to note that NOWS is a result of the abrupt discontinuation of opioid exposure that occurs at birth, and therefore is categorized as spontaneous opioid withdrawal. The use of *precipitated* withdrawal in research studies introduces a confounding variable, an opioid antagonist, to elicit withdrawal symptoms. Therefore, these models are not a true representation of the natural biological process of opioid withdrawal that neonates experience.

Years of research have allowed clinicians to understand the short-term withdrawal symptoms infants experience while going through opioid withdrawal. Similarly, researchers have been able to characterize the withdrawal symptoms in animal models of NOWS. Table 1.1 provides a summary of commonly observed withdrawal behaviors in rodent models of prenatal opioid exposure (Barr et al., 2011; Barr & Wang, 1992; Ceger & Kuhn, 2000; Fanselow & Cramer, 1988; Jones & Barr, 1995, 2000; Windh, Little, & Kuhn, 1995; Zhu & Barr, 2004). Symptoms associated with withdrawal are greatly influenced by age. For example, commonly observed behaviors seen in infant rats include paw movement, quietness, ultrasonic vocalizations, and togetherness (Barr et al., 2011). Whereas behaviors such as wet dog shakes, burrowing, and teeth chattering may not be observed until the second and third postnatal weeks and jumping, diarrhea occurring much later in life rat and mice (Fanselow & Cramer, 1988; Jones & Barr, 1995; Richardson et al., 2006).

Withdrawal Behaviors	Description
Burrowing	Sliding the body under the shavings of the observation chamber
Head swaying	Lateral and/or rotary motion of the head
Paw movement	Continuous movement of the hind paws without walking
Quiet	"Sedated" appearance without movement
Rolling	Turning the body over at least one full rotation
Ultrasonic vocalization	Vocalizations in the ultrasonic range (typically ~40 kHz) that imply distress in infant rodents
Walking/locomotion	Moving forward at least one step; Walking across cage
Wall climbing	Putting both forepaws on the wall of the observation chamber; typically with movement
Tremor	Mild lateral movements of the head that progress to a full-body lateral tremor
Mouthing	Opening and closing of the mouth, often accompanied by sucking sounds or rubbing of the mouth
Straub tail	Tail held erect at approximately 45-90 degrees from body
Stretching	Elongation of extremities
Paw fluttering/face cleaning/face swiping	Continuous movement of paws toward face
Vocalizations (audible)	Audible vocalizations
Wet dog shakes	Rapid shaking of the whole body
Jumps	Sudden leaping such that all four paws are off the bottom of the chamber
Togetherness	Bodily contact with one or more littermates
Teeth chatter	Movement of the jaw accompanied with constant chattering of the teeth
Rearing	Standing erect on two hind legs without leaning on wall
Diarrhea	Watery stool
Grooming	Licking any part of body, including face washing
Tail movement	Shaking, Twitching, Curling, or Sweeping movement of tail
Hind limb movement	Spontaneous shaking, tremor, or kicking of the hind limbs without full body movement

Table. 2.1 Opioid withdrawal behaviors in rodents
Commonly observed behaviors in rodent models of opioid withdrawal.

Development

Rodent studies have found prenatal opioid exposure leads to altered neurodevelopment and postnatal growth. Prenatal opioid exposure has been shown to lead to decreased brain mass as well as body weight in both male and female rats (Eriksson & Rönnbäck, 1989; Hung et al., 2013; Hutchings, Zmitrovich, Brake, Church, & Malowany, 1993; Wu et al., 2014). Additionally, there is evidence that prenatal opioid exposure leads to altered development of the nervous system in rodents. More specifically, neurons were found to have a decreased dendritic length and reduced spine density following prenatal morphine exposure in rats (Mei, Niu, Cao,

Huang, & Zhou, 2009; Ricalde & Hammer, 1990). Prenatal opioid exposure also led to decreased neurogenesis and altered postsynaptic activity within the striatum (Harlan & Song, 1994; Wu et al., 2014). Additionally, methadone exposure was found to affect the pattern of myelination in the early stages of brain development in rats. An increased expression of myelin associated proteins as well as an increase in axons with highly compacted myelin sheaths were found in pups 10-19 days-old (Vestal-Laborde et al., 2014). Similarly, low dose buprenorphine (0.3 mg/kg/day) exposure led to an increase in the expression of myelin basic protein and myelination in offspring. However, high dose buprenorphine (1.0 mg/kg/day) exposure led to a decrease in myeline basic protein expression as well as a decreased number of myelinated axons were observed in rats around one month of age. (Sanchez, Bigbee, Fobbs, Robinson, & Sato-Bigbee, 2008).

Prenatal opioid exposure can also disrupt several neurotransmitter systems including acetylcholine, dopamine, norepinephrine, and serotonin. A decrease in acetylcholine and choline acetyltransferase protein and mRNA as well as an increase in rate of turnover was found in rats prenatally exposed to opioids. (Guo, Enters, McDowell, & Robinson, 1990; Robinson, 2000). Changes in the mesolimbic dopamine pathways were observed in mice prenatally exposed to buprenorphine. An overall increase in excitatory synapses were found within the nucleus accumbens, prefrontal cortex, and anterior cingulate cortex (Boggess et al., 2020). Additionally, late gestational methadone exposure has been shown to result in a decrease in dopamine and norepinephrine levels within the forebrain of rat offspring during the early postnatal period. Norepinephrine levels increased by PND 40 in these offspring, however dopamine levels remained significantly lower (McGinty & Ford, 1980). Changes in turnover of norepinephrine was also observed within the hypothalamus following in utero opioid exposure in a gender

specific manner. A significant increase in norepinephrine turnover was observed in male offspring, whereas females experienced a decrease in norepinephrine turnover (Vathy, Rimanoczy, Eaton, & Katay, 1994). Additionally, increased noradrenergic activity was observed within the hippocampus of male rats following perinatal methadone exposure (Robinson, Maher, Wallace, & Kunko, 1997). A decrease in the 5-HT transport system was found within the cortex following in utero methadone exposure (Montis, Devoto, Angioi, Curreli, & Tagliamonte, 1983).

Opioids act on opioid receptors, μ (MOR), δ (DOR), κ (KOR), that are widely distributed throughout the central nervous system as well as peripheral tissues of the body. These receptors are naturally activated by three endogenous opioid peptides: β-endorphin, enkephalins, and dynorphins. Collectively opioid receptors and opioid peptides compose the endogenous opioid system which has been found to play a major role in response to the modulation of pain, reward, and stress responses (Benarroch, 2012). The endogenous opioid system is also known to play a role in regulating proper development of the nervous system (Hess & Zagon, 1988; Zagon, Tobias, & McLaughlin, 1997). Studies have found that prenatal opioid exposure leads to a dysregulation of the endogenous opioid system. More specifically, prenatal opioid exposure has been shown to decrease the number of MOR receptors within several areas of the brain including the striatum, thalamus, and amygdala (Belcheva et al., 1994; Chiou et al., 2003; Tempel et al., 1988). Similarly, a decrease in MOR binding and affinity was also observed in both the brain and spinal cord following prenatal opioid exposure during the early postnatal periods in rat offspring (Kirby, 1983; Kongstorp et al., 2020). Whereas, an increase in MOR binding was observed in adult rats. However, changes in MOR binding greatly differed between brain regions as well as between genders (Slamberova, Bar, et al., 2003; Slamberova,

Rimanoczy, Bar, Schindler, & Vathy, 2003; Slamberova, Rimanoczy, Cao, Schindler, & Vathy, 2005; Vathy, Slamberova, Rimanoczy, Riley, & Bar, 2003).

HPA and Immune system

Endogenous opioids are implicated in regulating the stress response and are known to affect the hypothalamic-pituitary-adrenal (HPA) axis as well as the autonomic nervous system (Drolet et al., 2001). Several studies have found prenatal opioid exposure to cause dysregulation of the HPA axis. Associated effects include a decreased release of adrenocorticotropin hormone (ACTH) following exposure to physiological and psychological stressors in adult offspring. However, basal levels of cortisol (CORT) and levels following exposure to stress were not affected (Rimanoczy, Slamberova, Riley, & Vathy, 2003; Slamberova, Rimanoczy, Riley, & Vathy, 2005). Additionally, a decrease in adrenal norepinephrine and epinephrine was found in adult rats following prenatal morphine exposure (Dutriez-Casteloot et al., 1999; Laborie et al., 2005). Altered activation of corticoid receptors has also been demonstrated in a rat model of prenatal opioid exposure. More specifically, prenatal morphine exposure prevented the upregulation of mineralocorticoid and glucocorticoid receptor binding following perinatal stress (Rimanoczy, Slamberova, Bar, & Vathy, 2006).

Although studies are limited, there is evidence that prenatal opioid exposure can also lead to altered immune function in offspring. A decreased fever response and increase in hypoalgesia was observed following LPS treatment in prenatally exposed rat and chick offspring (Schrott & Sparber, 2004; Shavit et al., 1998). Additionally, the cytotoxic activity of natural killer cells was decreased following prenatal morphine exposure in rats (Shavit et al., 1998). These findings suggest prenatal opioid exposure may have a lasting impact on the regulation of the HPA axis as

well as the immune system. Therefore, these offspring may be at higher risk for developing psychological or physiological disorders later in life.

Pain and Opioid-Induced Analgesia

Opioids are most commonly used for pain management, and induce analgesia by activating modulatory pain circuits withing the brain and spinal cord. These pain circuits originate within the midbrain and descend, by way of the rostral ventromedial medulla, to the dorsal horn of the spinal cord. Additionally, opioids produce analgesia by inhibiting ascending nociceptive pathways found within the dorsal horn of the spinal cord (Basbaum, 1984) . A number of studies have been conducted to determine how prenatal opioid exposure my affect analgesia and response to opioids later in life. Prenatal morphine exposure led to a heightened sensitivity to pain as well as a decrease in opioid sensitivity in adolescent offspring (Chiou et al., 2003; O'Callaghan & Holtzman, 1976; Tao, Chen, & Huang, 2011; Tao, Yeh, Su, & Wu, 2001; Timár et al., 2010). However, studies found that prenatal buprenorphine and methadone exposure led to increased sensitivity to pain without changes to opioid tolerance and sensitivity in adolescent rats (Chiang et al., 2015; Wallin et al., 2019). Additionally, an increase in opioid sensitivity has been observed in adults prenatally exposed to morphine (Biglarnia, Karami, & Hafshejani, 2013; Castellano & Ammassari-Teule, 1984; Chiang, Hung, Lee, Yan, & Ho, 2010; Gagin, Cohen, & Shavit, 1996). These studies suggest perception of pain and sensitivity to opioids are affected in offspring prenatally exposed to opioids in an age dependent manner with changes persisting into adulthood.

Seizure Activity

Seizures are a known symptom associated with the clinical presentation of NOWS (Kocherlakota, 2014). Prenatal opioid exposure has also been found to alter epileptic activity and seizure thresholds in offspring. A decrease in seizure threshold was observed in young (PND 15) rats prenatally exposed to morphine (Veliskova, Moshe, & Vathy, 1999). In contrast, an increase in seizure threshold was found in adults (Vathy, Velickova, & Moshe, 1998). In addition, prenatal morphine exposure led to an increase in seizure susceptibility in adolescent rats (Schindler, Velıskova, Slamberova, & Vathy, 2000) . However, a decrease in seizure susceptibility was observed in adults following in utero opioid exposure (Schindler, Slamberova, & Vathy, 2001). These studies provide evidence that infants prenatally exposed to opioids are not only at risk for seizures during the early postnatal period, but also may have increased risk for seizures later in life.

Reward

Opioids elicit their rewarding effects by activating the mesolimbic dopamine pathway found within the ventral tegmental area and nucleus accumbens leading to an increase in dopamine release (Wise & Bozarth, 1982). The rewarding effects of opioids have been well documented in adult models of opioid exposure. Additionally, there is growing evidence that prenatal opioid exposure leads to alteration in reward-related behavior as well as molecular changes in reward neurocircuitry. Previous studies have shown that changes in drug consumption, preference, sensitization, and tolerance were observed in offspring following prenatal opioid exposure (Grecco & Atwood, 2020). Several methods including operant intravenous self-administration, voluntary oral consumption, conditioned placed preference

(CPP), and locomotor sensitization assays have been used to determine changes in opioid sensitivity (Grecco & Atwood, 2020).

Self-administration and voluntary consumption are used to measure the strength of a drug's reinforcing actions (Sanchis-Segura & Spanagel, 2006). Several studies have found an increase in voluntary oral consumption and self-administration of opioids in adult offspring that were prenatally exposed to morphine (Glick, Strumpf, & Zimmerberg, 1977; Haydari et al., 2014; Riley & Vathy, 2006; Torabi, Pooriamehr, Bigdeli, & Miladi-Gorji, 2017). This suggests that prenatal opioid exposure increases the rewarding effects of opioids in these offspring.

Similarly, an increased preference for the drug paired environment was observed in adult rats following prenatal exposure to morphine during conditioned placed preference testing. Similar behavior was observed in rats prenatally exposed to methadone (Chiang et al., 2015; Gagin, Kook, Cohen, & Shavit, 1997; Riley & Vathy, 2006; Timár et al., 2010; Wu, Chen, Tao, & Huang, 2009). In addition, preference of the drug paired environment was also observed in young chicks (PND1) following prenatal morphine exposure (Wang et al., 2017). Interestingly, one study found that the timing of opioid exposure during gestation may play a role in these reinforcing effects of opioids. He et al. found that chicks exposed to morphine from E17-E20 experienced an increased preference for the morphine paired compartment. This preference was maintained over a 72-hour drug-free period. However, this conditioned place preference for the morphine paired compartment was not observed in chicks exposed to morphine on E5-E8, E9-E12, or E13-E16 (He et al., 2010). These findings suggest offspring prenatally exposed to opioids have increased sensitivity to the positive reinforcing properties of opioids that persist from the early postnatal period into adulthood.

Previous studies have shown that repeated opioid exposure results in locomotor sensitization which is evidenced by a consistent increase in motor activity (Sanchis-Segura & Spanagel, 2006; Wise & Bozarth, 1987). Sanchis-Segura and Spanagel suggest that locomotor sensitization is a reflection of drug "wanting" and associated with an increase in drug seeking behavior (Sanchis-Segura & Spanagel, 2006). Previously, these behaviors were studied in adult models of opioid use. However, there are emerging studies that have found this locomotor sensitization in offspring that were prenatally exposed to opioids. Hyperlocomotion has been observed in rat, mouse, and chick models of prenatal opioid exposure (Kvello et al., 2018; Wang et al., 2017; Wu et al., 2009). These findings suggest prenatal opioid exposure may alter development of reward pathways within the brain leading to altered reward-related behaviors. Consequently, prenatally exposed infants may be at greater risk for developing drug-seeking and drug-taking behavior later in life.

Anxiety and Depression

Studies have also found evidence of anxiety and depression in offspring that were prenatally exposed to opioids. Using an elevated plus maze to evaluate anxiety-like behaviors, researchers found that adult rats prenatally exposed to morphine spent less time in the open arms of the maze compared to control offspring (Ahmadalipour et al., 2015; Klausz et al., 2011). Additionally, these offspring spent less time in the lit compartment of a light/dark box indicating anxiety like behavior (Ahmadalipour et al., 2015). Similar behaviors were also observed in rats prenatally exposed to methadone and buprenorphine. However, the effects of methadone and buprenorphine on anxiety-like behaviors were less pronounced compared to morphine exposed offspring (Chen et al., 2015). Studies evaluating the effect of prenatal opioid exposure on the

development of depressive-like behaviors found a decrease in escape-oriented behaviors during forced swimming and tail suspension tests in adolescent rats prenatally exposed to buprenorphine (Hung et al., 2013; Klausz et al., 2011; Wu et al., 2014). Collectively, these studies demonstrate that infants prenatally exposed to opioids may be at greater risk for developing anxiety and depression later in life.

Learning and Memory

Clinical studies have suggested that prenatal opioid exposure may lead to cognitive deficits. However, there are many conflicting studies on this matter. Additionally, the severity of cognitive impairments, as well as how long they will persist, in children diagnosed with NOWS is still unclear. The unknown long-term consequences of NOWS on cognition have led many researchers to examine the effects of prenatal opioid exposure on learning and memory. Several studies have been conducted to understand the neural effects of prenatal opioid exposure within hippocampus. The endogenous opioid system is a known regulator of neuronal activity and neurogenesis within the hippocampus (Drake, Chavkin, & Milner, 2007; Sargeant, Miller, & Day, 2008; Simmons & Chavkin, 1996; Zhang, Loh, & Law, 2016). As mentioned above, exogenous opioids are known to modulate the activity of the opioid system. Therefore, it is likely that prenatal opioid exposure may impact hippocampal development as well as cognition.

Several studies have found impaired spatial learning in rodents following prenatal exposure to morphine and oxycodone. This was made evident by an increase in reference and working memory errors when assessed using the Y-maze, Morris water maze, and radial arm maze (Davis et al., 2010; Lin et al., 2009; Niu et al., 2009). These deficits were observed in both adolescent and adult offspring. However, prenatal methadone exposure did not have any effect

on spatial learning in rats at PND 1, PND 30, or adulthood (Chiang et al., 2015). In addition to deficits in spatial memory, deficits in passive avoidance memory have also been observed in adolescent and adult rodents following prenatal morphine exposure (Ahmadalipour et al., 2015; Nasiraei-Moghadam et al., 2013). Similarly, deficits in passive avoidance learning and memory were also observed in chicks during the early postnatal period after being prenatally exposed to morphine (Che et al., 2005; Jiang et al., 2011; Wang et al., 2017a; Wang et al., 2017b).

Deficits in learning and memory behaviors associated with prenatal opioid exposure have been linked to several cellular changes within the hippocampus. A decrease in long term potentiation (LTP) , long term depression (LTD), and synaptic plasticity have been observed in rodents prenatally exposed to morphine, but not methadone (Chiang et al., 2015; Jiang et al., 2011; Niu et al., 2009; Tan et al., 2015; Velisek, Slamberova, & Vathy, 2003; Villarreal, Derrick, & Vathy, 2008; Yang et al., 2003). A decrease in GABA-containing neurons was associated with the decrease in LTP (Niu et al., 2009). An increase in proteins associated with apoptosis such as Bax/Bcl-2 as well as increased capsase-3 activity were found in hippocampal tissue taken from adolescent and adult rats following in utero morphine exposure (Nasiraei-Moghadam et al., 2013). Additionally, a decrease in BDNF expression was found in adolescent and adult rats that were prenatally exposed to morphine (Ahmadalipour et al., 2015; Nasiraei-Moghadam et al., 2013). Prenatal morphine exposure has also been shown to lead to altered kinetic properties of NMDA receptors as well as a decrease in the mRNA and protein expression of the NMDA receptor subunits NR1, NR2A, and NR2B (Lin et al., 2009; Yang et al., 2000). Similarly, a decrease in PSD-95 was also observed in these rats (Lin et al., 2009). Collectively these studies provide evidence that prenatal opioid exposure can lead to cellular changes within the

hippocampus that are associated with impairments in learning and memory in offspring that can extend from adolescence into adulthood.

CONCLUSION

The lasting effects of prenatal opioid exposure and withdrawal on children is still unclear. Therefore, the use of animal research is still needed to help us understand the long-term consequences of NOWS. Over the past several decades, numerous animal studies have been conducted to understand both the short and long-term effects of prenatal opioid exposure on the developing fetus. Collectively, studies have demonstrated that prenatal opioid exposure can lead to several neurodevelopmental effects including altered postnatal development, increased reward seeking behavior, altered immune response, increased seizure susceptibility, and decreased learning and memory in offspring. However, the large amount of inconsistences within these studies limits the generalization and translatability of preclinical findings. Additionally, differences in in utero brain development between commonly used animals and humans also limits the translatability of current animal models. Therefore, a more clinically translatable model of prenatal opioid exposure is needed to help us understand the consequences of NOWS.

CHAPTER 3

SPINY MICE

INTRODUCTION

Spiny mice (*Acomys* spp.) collectively refers to several rodent species belonging to the genus *Acomys*, that are native to regions across Africa, the Middle East, and parts of southern Asia (Haughton, Gawriluk, & Seifert, 2016; Nowak, 1999). My research focused on one of these species, *Acomys cahirinus*. This species has been found to inhabit the deserts of Egypt and Israel concentrated to areas of rocky terrain such as rock canyons and near cliffs. Spiny mice received their common name because of the spiny hairs that are found covering their dorsum (Nowak, 1999). These rodents have been used to investigate many biological processes including mature-onset and diet-induced diabetes, parental and neonatal behaviors, skin regeneration, and late gestational development (Brunjes, 1990; Creutzfeldt, Mende, Willms, & Söling, 1970; Gonet, Stauffacher, Pictet, & Renold, 1966; Porter & Doane, 1976; Porter, Tepper, & White, 1981; Seifert et al., 2012; Shafrir, 2000). Here we discuss the unique biological characteristics found in spiny mice, and review their use in perinatal research.

TAXONOMY

Acomys spp. belong to the family Muridae, and were originally thought to be included within the Murinae subfamily alongside the Old-World rodents mouse (Mus) and rats (Rattus) based on dental structure analysis (Steppan, Adkins, & Anderson, 2004; Steppan, Adkins, Spinks, & Hale, 2005) . However, it was later proposed that Acomys may not be directly related to true murines (Sarich, 1985). Instead, it was found that Acomys hold a closer phylogenetic relationship to the Mongolian gerbils (*Meriones unguiculatus*) than mice (*Mus musculus*),

suggesting that spiny mice may belong within the subfamily Gerbillinae instead of the Murniae subfamily (Agulnik & Silver, 1996). Molecular studies found that *Acomys* along with the *Uranomys*, *Lophuromys*, and *Deomys* all share a common ancestor with gerbils and belong to the subfamily Deomyinae, a sister clade to Gerbillinae (Michaux, Reyes, & Catzeflis, 2001). Collectively, members of Deomyinae and Gerbillinae share a common ancestor with the Murinae and together make up the family Muridae (Steppan et al., 2004, 2005). A summary of this phylogenetic information can be seen in Figure 3.1 (Bellofiore & Evans, 2019; Michaux et al., 2001).

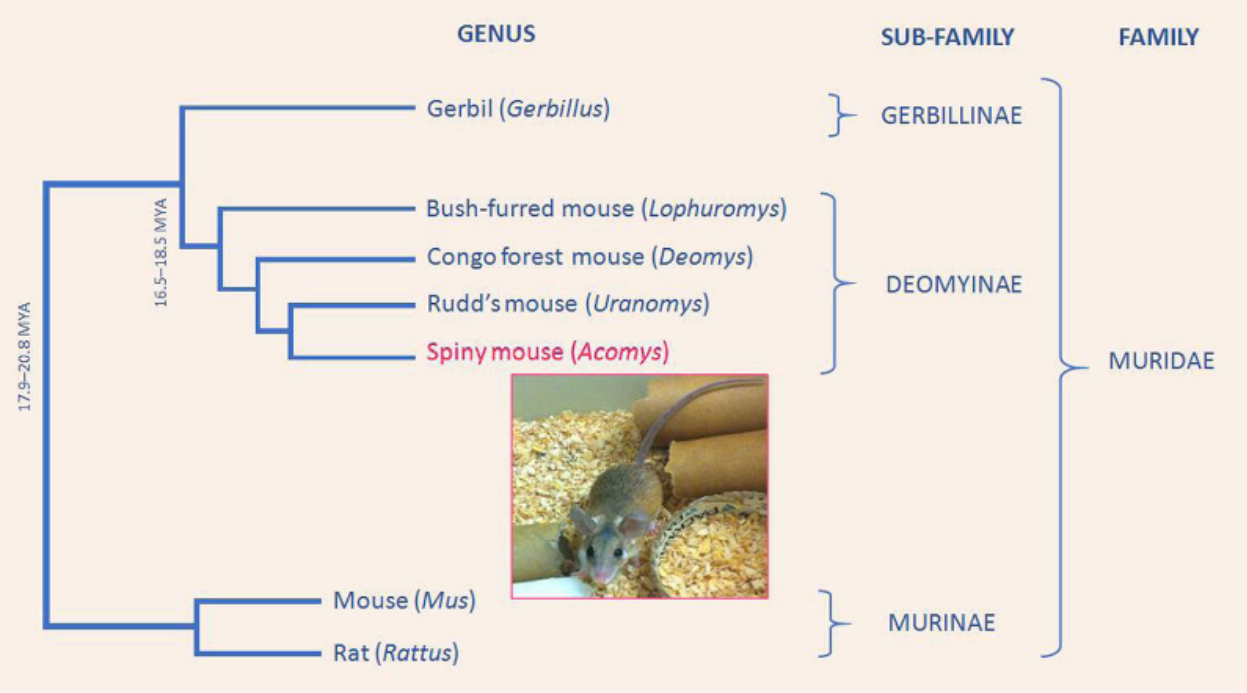

Fig. 3.1 Phylogenetic tree demonstrating evolution of spiny mouse (red) in relation to other murids.
Controversy of classification of this species has since been resolved using LCAT and vWF genetic sequencing and confirms *Acomys* belongs to the Deomyinae subfamily, a sister clade of Gerbillinae, as opposed to the Murinae (Old World mice and rats). Image adapted from Evolutionary History of the Most Specious Mammals: Molecular Phylogeny of Muroid Rodents (Michaux et al., 2001). (Bellofiore & Evans, 2019) Reprinted by permission from Springer Nature: Springer Nature. Journal of Assisted Reproduction and Genetics. Monkeys, mice and menses: the bloody anomaly of the spiny mouse. Bellofiore & Evans, © 2019.

LIFE CYCLE

Spiny mice have a life expectancy of 2-4 years, however there is evidence that spiny mice can live up to 7 years in captivity (Bodenheimer, 1949; Haughton et al., 2016; Morrison, Dieterich, & Preston, 1977). Spiny mice are considered sexually mature by 2-3 months of age, and females in our colony were observed to undergo continual mating and offspring production throughout life (Young, 1976). Sexual maturity coincides with the emergence of the characteristic golden spiny hairs found across the dorsum of adult spiny mice (Fig. 3.2) (Haughton et al., 2016; Montandon, Tzika, Martins, Chopard, & Milinkovitch, 2014). Seasonal breeding is reported in spiny mice, with the preference for breeding in the spring and summer months (Bodenheimer, 1949; Delaney & Happold, 1979; Derrickson, Jerrard, & Oftedal, 1996). However, we were able to remedy this with the addition of supplemental heating and lighting provided by daily heat lamp exposure. Successful breeding was observed in female spiny mice that were more than three years of age. The long-life expectancy of these mice makes them ideal for long term studies and aging related research.

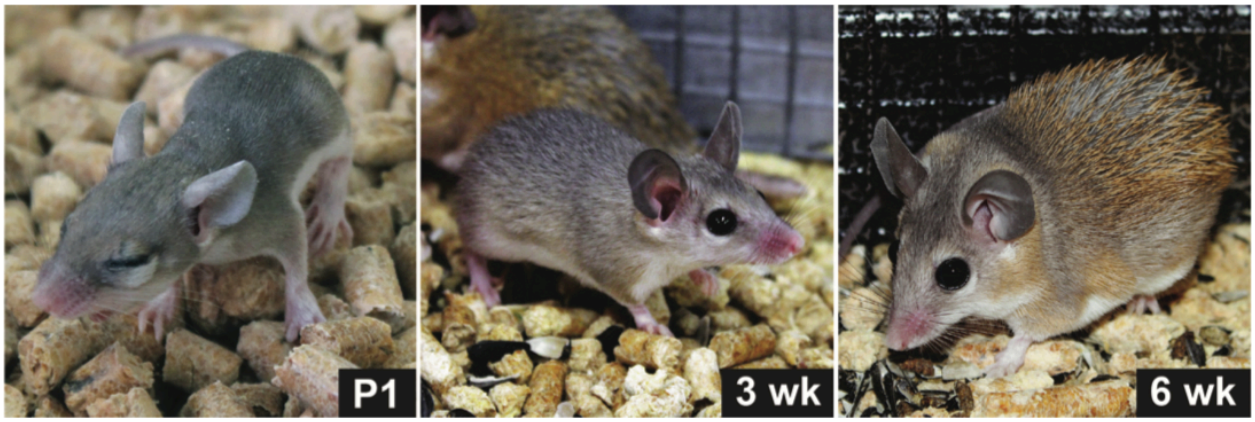

Fig. 3.2 Postnatal development of *Acomys cahirinus*
A newborn *A. cahirinus* (P1) demonstrating precocial development. Note open eyes, unfurled ears, and hair coat. In this 3-wk old *A. cahirinus*, adult coat color is visible at the boundary between the white underside and gray, juvenile coat. The spiny hairs have emerged from the dorsal skin of this 6-wk old *A. cahirinus*. (Haughton et al., 2016) Reprinted by permission from American Association for Laboratory Animal Science: American Association for Laboratory Animal Science. Journal of the American Association for Laboratory Animal Science. The Biology and Husbandry of the African Spiny Mouse (*Acomys cahirinus*) and the Research Uses of a Laboratory Colony. Haughton et al., © 2016.

UNIQUE BIOLOGICAL CHARACTERISTICS

Development

Although closely related to the rat and mouse, spiny mice have been found to possess several unique biological characteristics allowing for them to be utilized in a diverse array of research. One such characteristic is their lengthy gestational period which last on average 38-39 days (Dickinson & Walker, 2007). With a gestational period of nearly two times the length of other rodent species, spiny mice experience extensive in utero organogenesis. More specifically, research has shown that the liver, kidney, lung, and regions of the brain undergo substantial development in utero and are nearly complete at time of birth in these mice (Brunjes, Korol, & Stern, 1989a; Dickinson, Walker, Cullen-McEwen, Wintour, & Moritz, 2005a; Lamers, Mooren, Graaf, & Charles, 1985; Oosterhuis, Mooren, Charles, & Lamers, 1984). The progression of in

utero development of spiny mice is seen in Fig. 3.3 (Brunjes, 1990). The rate of in utero organogenesis has been found to more closely resemble human fetal development compared to other rodents currently used in perinatal research.

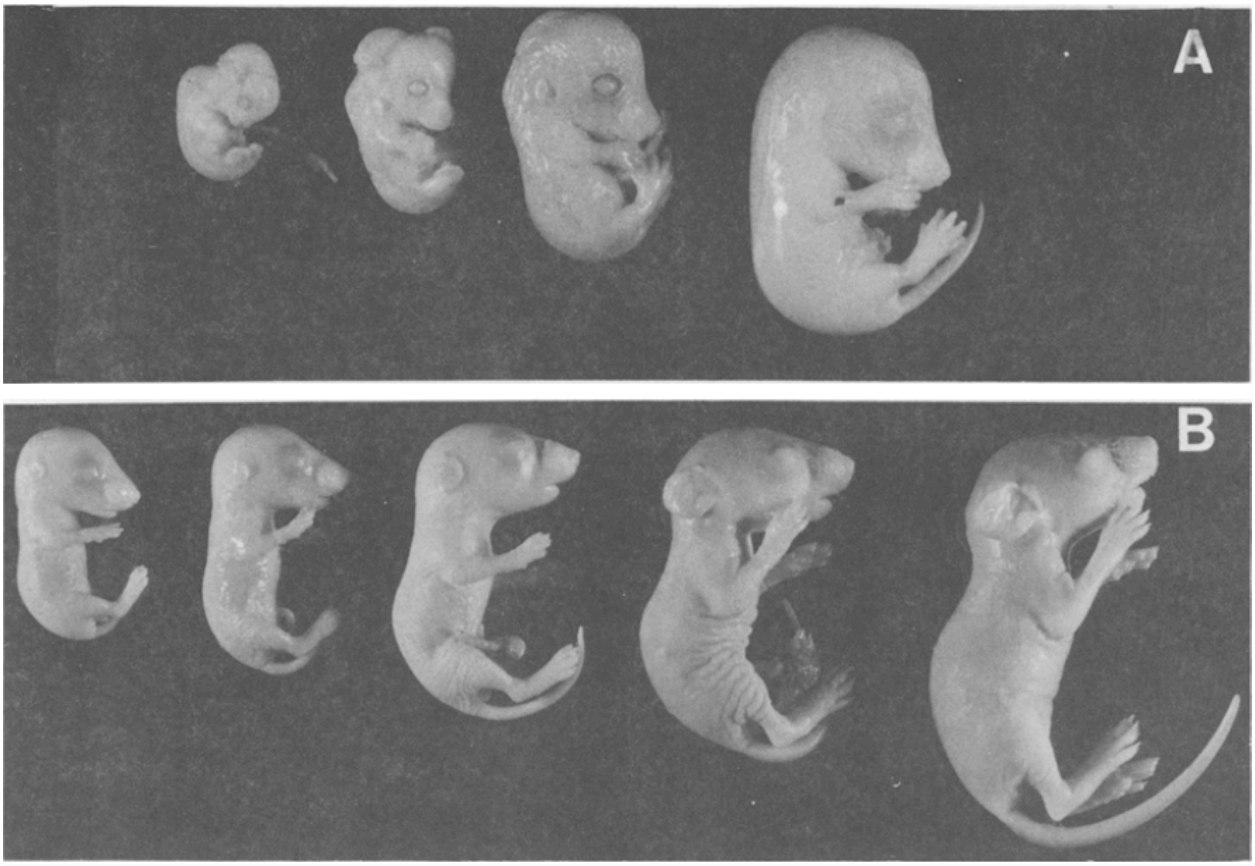

Fig. 3.3 In utero development of *Acomys cahirinus* fetus
(A) Left to right: Embryonic Days 16, 18 (with deformed cranium), 20, 22 (B) Left to right: Embryonic Days 24, 26, 28, 30, 34. (Brunjes, 1990) Reprinted by permission from Springer Nature: Springer Nature. Psychobiology. The precocial mouse, Acomys Cahirinus. Brunjes, © 1990.

The advanced in utero development of spiny mice is much different compared to other rodent species which experience extensive development and maturation postnatally during the

early neonatal period. Developmental differences between spiny mice pups and the common laboratory rat (Rattus norvegicus) can be seen in Fig. 3.4 (Brunjes, 1990). A deeper examination of gestational development revealed that spiny mice develop more slowly in utero compared to the laboratory rat. With approximately a 2-day delay observed at E14, 5 days at E22, and by E26 the spiny mouse is more than 7.5 days less mature than the rat (Dieterlen, 1963). Spiny mice give birth to relatively small litters typically consisting of 1-4 pups, with an average litter size of 2-3. However, in rare cases spiny mice can have litters with up to 5 pups (Dickinson & Walker, 2007). It is thought that the small litter sizes observed in spiny mice allows for the increased growth and advanced in utero development of spiny mice offspring.

Fig. 3.4 Comparison of newborn *Acomys cahirinus* and rat
Developmental comparison of newborn *Acomys cahirinus* (left) and the common laboratory rat (Rattus norvegicus) (right). (Brunjes, 1990) Reprinted by permission from Springer Nature: Springer Nature. Psychobiology. The precocial mouse, Acomys Cahirinus. Brunjes, © 1990.

Spiny mice are a precocial species, which is thought to be one of their most unique characteristics. At birth, they are covered in a fine grey fur, eyes open, and ears unfolded (Fig.

3.2) (Brunjes, 1990; Haughton et al., 2016). Due to their advanced development, newborn pups are also capable of thermoregulation and are able to walk and self-feed shortly after birth (D'Udine, Gerosa, & Drewett, 1980; Flückiger & Operschall, 1962). This is much different from commonly used laboratory rodents that are altricial species which do not achieve this level of development until approximately the second to third postnatal week. These characteristics are thought to be an evolutionary adaptation to the hostile arid environments of the desert where spiny mice are native to. This early independence observed in young pups is thought to allow for escape from predators (Brunjes, 1990). Spiny mice pups are known to be extremely curious and social during early postnatal days compared to other commonly used laboratory rodents (Ratnayake, Quinn, Daruwalla, Dickinson, & Walker, 2014). These behavioral attributes paired with their early mobility allow for behavioral tests and assessments to be conducted in spiny mice pups, that would typically be reserved to adolescent and adults in other rodent species.

Of particular interest to my research, is the brain development of spiny mice. Several studies have been conducted over the years to understand brain development in spiny mice and how it relates to the precocial nature of this species. Examination of Nissl-stained tissue taken from the developing hippocampus, visual cortex, and olfactory bulb showed a slower development of these brain regions in spiny mice compared to rat. More specifically, by E14 the regions of the telencephalon and visual cortex were found to be approximately 2 days behind in rats of the same post-conception age. This gap grew to almost a six-day difference on E28 in spiny mice, with the rat brain observed to be more mature at this age (Brunjes, 1989). Differences in the onset of neurogenesis within the telencephalon were also observed between spiny mice and rat with cell production beginning later in gestation and lasting longer than observed in rats (Brunjes et al., 1989a). Spiny mice were also found to have a significantly

higher level of cholinergic activity at birth compared to rats (Pintor, Alleva, & Michalek, 1986). Additionally, spiny mice experience a growth spurt in brain development around the time of birth which is much sooner than other rodents like the rat and mouse, and more comparable to human brain development (Fig. 3.5) (Quinn, Ratnayake, Dickinson, Castillo-Melendez, & Walker, 2016b). These findings highlight the fact that midgestational brain growth is much slower in spiny mice compared to other rodents, allowing for a more in depth look into early brain development (Brunjes, 1990).

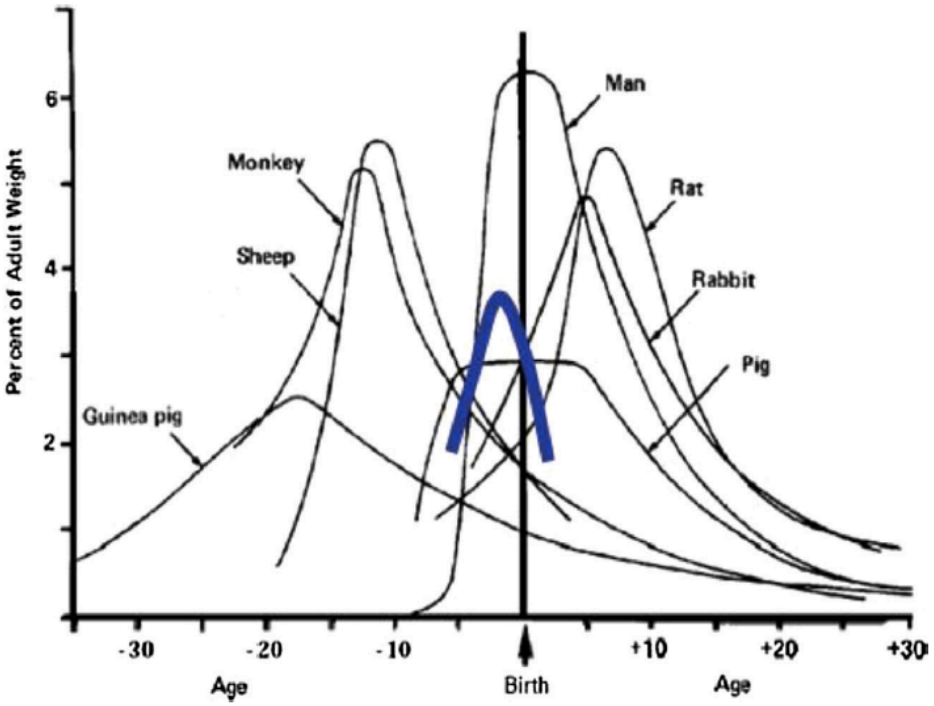

Fig. 3.5 Comparing brain growth between species
The brain growth spurts of seven mammalian species expressed as first order velocity curves of the increase in weight with age. Each unit of time for each species represents: guinea pig: 1 day; rhesus monkey: 4 days; sheep: 5 days; pig: 1 week; man: 1 month; rabbit: 2 days; rat: 1 day; spiny mouse (indicated in blue): 2.5 days. Of significance in that both humans and the spiny mouse appear to have brain growth spurts, relative to body weight, around the time of birth. In contrast, monkeys, sheep, and guinea pigs have brain growth spurts before birth in mid to late gestation, whereas rats, rabbits, and pigs have a grown spurt postnatally. This suggests that the spiny mouse may be a more suitable animal model of brain development compared with some other species. Adapted from (John Dobbing & Sands, 1979). (Quinn, Ratnayake, Dickinson, Castillo-Melendez, & Walker, 2016c) Reprinted by permission from Elsevier: Elsevier. The Journal of Steroid Biochemistry and Molecular Biology. The feto-placental unit, and potential roles of dehydroepiandrosterone (DHEA) in prenatal and postnatal brain development: A re-examination using the spiny mouse. Quinn et al., © 2016.

Similar to prenatal brain development, differences in postnatal brain development have been found between spiny mice and rats. Due to their precocial nature, it was originally assumed that spiny mice were born after the spike in postnatal brain growth observed in other altricial species such as rat and mouse. However, it was found that despite the increased in utero development of the brain, spiny mice also experience a period of rapid brain growth after birth as

seen in other rodents as well as humans (Brunjes et al., 1989a). More specifically, regions of the brain such as the olfactory bulb, hippocampus, and neocortex experience postnatal growth (Brunjes, 1983; Brunjes, 1984; Brunjes, 1985). Additionally, Brunjes found that spiny mice experience a more rapid volumetric growth in the hippocampus, olfactory bulb, and myelination compared to mice and rats at the same post-conception age (Brunjes, 1983; Brunjes, 1984; Tessitore & Brunjes, 1988). These regions undergo a rapid phase of growth during the first 20 postnatal days. For example, the olfactory bulb at birth is approximately 50% of the size observed in adult spiny mice at PND 60 and grows to nearly 90% of the adult size by PND 10. Similarly, at birth, the hippocampus is also about 50% of the adult size, however growth occurs much slower than other brain regions, as it reaches approximately 70% of the adult size by PND 70. Lastly, the cerebral cortex is approximately 70% of its adult size at birth and undergoes rapid growth by PND 10. However, this pattern of rapid volumetric growth was not observed in all regions of the brain as development of the visual cortex is similar between spiny mice and the common laboratory mouse (Brunjes, 1985). As shown in Fig. 3.6, adult spiny mice have an overall must larger brain compared to C57bl/6 mice. Interestingly, spiny mice have a relatively thin cortex compared with other species and possess a much larger hippocampus compared to rats (D'Udine & Alleva, 1988; D'udine & Gozzo, 1983). Collectively, these findings suggest that spiny mice are a unique rodent species with in utero brain development that more closely resembles that seen in humans and possess many differences in postnatal development from the commonly used laboratory rats and mice.

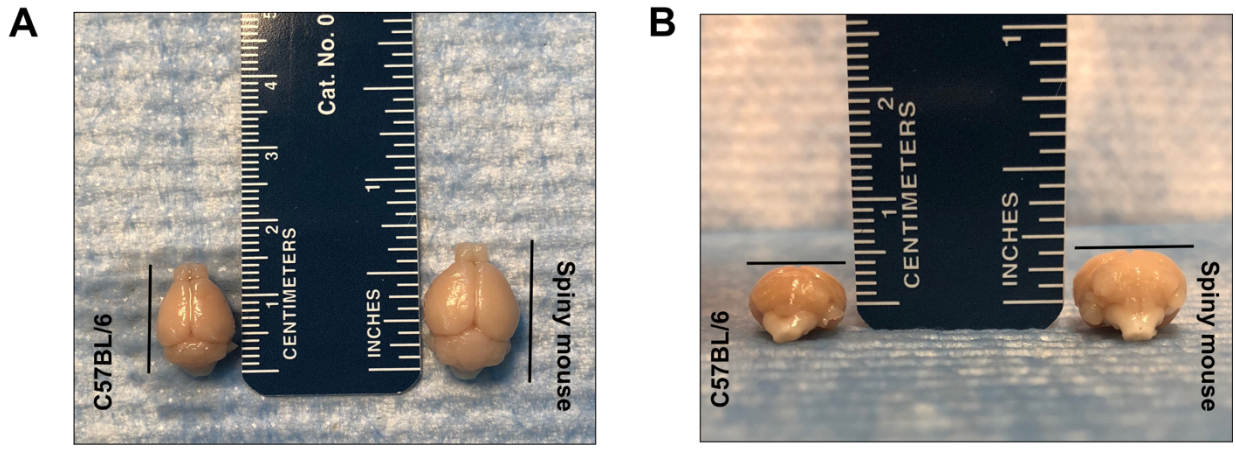

Fig. 3.6 Size comparison of length (A) and height (B) of age matched 4-month C57bl/6 and spiny mouse brains

Hormone and Enzyme Expression

Spiny mice possess a unique hormonal profile and enzyme expression. In contrast to most rodents which have corticosterone as their primary circulating glucocorticoid, spiny mice synthesize cortisol (Lamers et al., 1986). It was also found that spiny mice express the enzyme cytochrome P450 17- α -hydroxylase (CYP17) which is needed for dehydroepiandrosterone (DHEA) synthesis (Quinn et al., 2013). CYP17 is not found in other commonly used laboratory rodents, therefore they are unable to synthesize DHEA. It was later found that DHEA is synthesized in the brain and adrenal glands of spiny mice (Quinn et al., 2014; Quinn, Ratnayake, Dickinson, Castillo-Melendez, & Walker, 2016a; Quinn et al., 2013). DHEA is the primary carbon-19 steroid produced in humans and mammals by the adrenal gland and is an important precursor to sex steroid biosynthesis (Alonso, 2000; Parker, 1999). It has also been shown to be involved in several physiological systems including the nervous, immune, and somatic growth and development systems (Arquitt, Stoecker, Hermann, & Winterfeldt, 1991; Chen & Parker,

2004; Kroboth, Salek, Pittenger, Fabian, & Frye, 1999; Zemel & Katz, 1986). Researchers found that DHEA, cortisol, and aldosterone were all present in fetal circulation by G30 in spiny mice. At birth, circulating levels of DHEA in spiny mice were found to be comparable to concentrations observed in human infants (Quinn et al., 2013). As seen in humans, concentrations of DHEA fluctuate in aging spiny mice with decreased levels observed in advanced age (Quinn et al., 2016b). This is of significant interest to researchers, as the human temporal pattern of DHEA biosynthesis has not been identified in any other species except for the chimpanzee (Cutler et al., 1978; Quinn et al., 2016b; Smail, Faiman, Hobson, Fuller, & Winter, 1982). The structure of the spiny mouse fetal adrenal gland is developmentally, morphologically, and biochemically comparable to the human and fetal primate (Quinn et al., 2016c). Additionally, endogenous hormone levels of cortisol, glucagon, and insulin in fetal spiny mice were found to mimic human fetal concentrations more closely than the rat or mouse (Dobbing, 1971). Collectively, these findings suggest that spiny mice possess the unique capability to produce hormones and enzymes not typically seen in other rodents. However, these substances are synthesized in humans, making spiny mice a more suitable rodent to utilize for preclinical research.

Menstruation

Spiny mice were recently identified as the only known rodent species to experience menstruation (Bellofiore et al., 2016). Approximately 98% of all mammalian species do not experience menses (Bellofiore et al., 2016). Menstruation is thought to be limited to higher order primates, including humans and monkeys, as well as a few species of bats, and the elephant shrew (Van Der Horst and Gillman, 1941; Emera, Romero, & Wagner, 2012; Rasweiler &

Bonilla, 1992; Rasweiler, 1991; Zhang et al., 2007). Additionally, less than 0.9% of menstruating species are non-primates, making spiny mice an extremely rare rodent (Bellofiore et al., 2016).

Menstruation is the cyclical shedding of endometrium that occurs in the absence of pregnancy, which is accompanied by bleeding in the uterine cavity and vagina. This process is much different than the estrous cycles observed in most mammals including the common lab mouse and rat. In these rodents, exogenous signals such as changes in temperature and rainfall associated with seasonal changes, male pheromones, or mechanoreceptor stimulation that occurs during coitus can lead to ovulation (Downey, 1980). Instead, spiny mice undergo spontaneous and cyclical ovulation throughout the year (Bellofiore et al., 2016).

Menses were detected in virgin female spiny mice via vaginal lavage. The length of their menstrual cycle ranges from 6-10 days with an average cycle lasting 9 days and experience a menstrual period lasting on average 3 days. Despite the shorter menstrual cycle, the menstrual period accounts for 20-40% of the total menstrual cycle in spiny mice, which is comparable to the 15-35% seen in women (Bellofiore et al., 2016). These findings were the first to report evidence of a rodent capable of experiencing menstruation. A postpartum estrous also occurs in female spiny mice which is helpful in determining gestational age in subsequent litters (Dieterlen, 1961, 1962; Peitz, 1981). In addition, symptoms of premenstrual syndrome (PMS) have been observed in spiny mice, giving them the potential to serve as a natural model of PMS in the future (Bellofiore & Evans, 2019). The menstrual cycle coupled with small litter sizes, increased in utero organogenesis, and unique hormonal profiles has led to the increased interest in spiny mice for use in perinatal and women's reproductive health and fertility research.

SPINY MICE IN PERINATAL RESEARCH

Due to their lengthened gestation, small litter size, and increased in utero organogenesis, spiny mice are recognized as an ideal model for perinatal research. To date, spiny mice have been utilized to model and study fetal growth restriction, maternal immune activation, birth asphyxia and perinatal hypoxia. Additionally, understanding of the discoid haemotrichorial placenta, and its involvement in the feto-placental unit has been well researched in spiny mice. These studies further highlight the importance and uniqueness spiny mice offer the world of prenatal research and the search for a more translatable model for human in utero development.

Ireland et al. were the first to develop a model of near-term birth asphyxia in spiny mice and showed that a short hypoxic episode of 7.5 minute at gestational day 37 led to a significant decrease in neonatal survival (Ireland, Dickinson, Snow, & Walker, 2008). Surviving asphyxiated pups showed increased inflammation and apoptosis within the central nervous system during the first postnatal week. A significant impairment in non-spatial memory and learning tasks were also observed at one month of age and mild neurological deficits persisted throughout the first two postnatal weeks (Hutton, Ratnayake, Shields, & Walker, 2009; Ireland, Dickinson, Fleiss, Hutton, & Walker, 2009). It was later found that global fetal asphyxia led to sex-dependent changes in hippocampal structure and function of spiny mice (Fleiss et al., 2011).

This model of developmental brain injury has been used to investigate the efficacy of maternally administered neuroprotective agents to diminish the effects of neonatal hypoxic injuries. Hutton et al. identified melatonin as a possible prophylactic agent to protect against hypoxic-ischemic brain injury at birth. Asphyxiated pups exposed to melatonin in utero showed a significant decrease in inflammation and cellular apoptosis in the CNS compared to control pups (Hutton, Abbass, Dickinson, Ireland, & Walker, 2009). Maternal administration of the

endogenous steroid allopregnanolone led to a greater rate of survival in asphyxiated pups and prevented the decrease in long term potentiation and increase calcium channel expression associated with birth asphyxia. However, prenatal allopregnanolone exposure also led to a decrease in cell proliferation within the developing hippocampus suggesting that allopregnanolone may have a detrimental effect on postnatal hippocampal dependent behaviors (Fleiss et al., 2011). Additionally, maternal creatine supplementation was found to increase survival rates and protect the developing diaphragm, kidneys, and brain from the damaging effects of hypoxia-induced damage associated with near-birth asphyxia (Cannata et al., 2010; Ellery et al., 2013; Ireland, Castillo-Melendez, Dickinson, Snow, & Walker, 2011; Ireland et al., 2008; LaRosa et al., 2016). Most recently, a comparative study of activated protein C (APC) and a variant form 3K3A-APC revealed that 3K3A-APC was able to diminish the neuro-inflammatory response and cell death within deep grey matter and the hippocampus following asphyxia suggesting it may be a useful therapy to reduce neonatal ischemic brain injuries associated with hypoxia (Ellery et al., 2019).

Spiny mice have also been used to model maternal immune activation which is associated with the development of mental illness disorders such as schizophrenia and autism in children and adolescents born to mothers who suffered an infection during pregnancy (Brown, 2006; Brown et al., 2004, 2005; Brown, Cohen, Greenwald, & Susser, 2000; Brown, Schaefer, et al., 2000). Prenatal exposure to viral mimetic poly I:C at mid-gestation (G20) led to a significant impairment in non-spatial learning and memory tasks as well as a decrease in motor activity. Evidence of astrogliosis and microglial activation as well as decreased reelin positive cells within the hippocampus were observed in these mice at PND 1 and PND 100, suggesting that maternal immune activation has long lasting effects on brain development that extends into

adulthood in spiny mice. These changes were associated with impaired non-spatial memory and learning tasks and decreased motor activity in juvenile (3 weeks) and post-pubescent (9 weeks) spiny mice (Ratnayake, Quinn, Castillo-Melendez, Dickinson, & Walker, 2012). Surprisingly, maternal immune activation led to a significant decrease in proinflammatory cytokines in the fetal brain during the first 24 hrs following treatment in spiny mice. However, poly I:C exposure had a significant impact on behavior in juvenile offspring evidenced by abnormal sensorimotor gating, a decrease in social interaction with other mice, and impaired learning and memory (Ratnayake, Quinn, LaRosa, Dickinson, & Walker, 2014). Despite the early decrease in pro-inflammatory cytokines, further studies suggest that maternal immune activation at G20 may lead to a delayed neuroinflammatory response observed in pre-pubescent spiny mice and is mediated by the pro-inflammatory transcription factor, NF-κB1, and the epidermal growth factor system (Ketharanathan, Pereira, Reets, Walker, & Sundram, 2021). These studies provide evidence that maternal immune activation in spiny mice, a rodent species that more closely models human brain development, leads to abnormal brain development and behavior that lasts into adolescence and adulthood.

Elevated glucocorticoids during pregnancy brought on by increased stress and maternal malnutrition has been shown to have detrimental effects on the in utero environment and the developing fetus (Dickinson & Wintour, 2007). These effects are thought to increase the risk of developing disorders such as diabetes, cardiovascular disease, and metabolic syndrome (Gluckman, Cutfield, Hofman, & Hanson, 2005; Mcmillen & Robinson, 2005; Vehaskari & Woods, 2005). Spiny mice have been used in several studies to assess the effects of maternal glucocorticoid exposure by administering dexamethasone, a synthetic glucocorticoid, via osmotic pump from G20-G23 in pregnant spiny mice. This brief glucocorticoid exposure was

found to lead to an altered renal gene expression in the fetal kidney and a decreased number of nephrons in adult spiny mice offspring. However, these changes had no effect on blood pressure in these mice suggesting that decreased nephron number does not definitively result in development of hypertension (Dickinson, Walker, Wintour, & Moritz, 2007)

Subsequent studies focused on the effects of maternal glucocorticoid exposure on the placenta. O'Connell et al. found that the placental response to excess maternal glucocorticoid exposure differed between genders. Dexamethasone exposure led to an altered placental structure that was evident immediately following exposure and persisted for two weeks. A reduction in placental mRNA expression was observed in both male and female fetuses immediately after exposure, however when assessed at G37 (two weeks after exposure), differences in mRNA among male and female placentas were observed. These changes were suggested to have a lasting effect on fetal development with differences in male and female offspring (O'Connell, Moritz, Roberts, Walker, & Dickinson, 2011). Additionally, in utero dexamethasone exposure led to decreased branching morphogenesis which may lead to altered vascularization of the placenta. Gender differences in the spatio-temporal expression of genes associated with branching morphogenesis and placental glycogen storage were also observed in spiny mice offspring (O'Connell, Moritz, Walker, & Dickinson, 2013; O'Connell et al., 2011). These studies suggested that the effects of maternal glucocorticoid exposure were greater in male fetal spiny mice compared to females suggesting males are at greater risk for adverse developmental outcomes following exposure to a suboptimal in utero environment (O'Connell et al., 2011; O'Connell, Moritz, Walker, & Dickinson, 2013).

CONCLUSION

Collectively these studies present spiny mice as a unique rodent species for perinatal research. Here we propose the use of spiny mice to assess the short- and long-term effects of in utero opioid exposure. Due to their lengthened gestational period, these mice experience increased in utero organogenesis and exhibit similar brain development patterns as humans. This makes them a more suitable species to understand the effects of in utero opioid exposure on the developing brain. Additionally, their advanced development and precocial birth allows for characterization of unique withdrawal symptoms associated with in utero opioid exposure. We are hopeful that this study will help us to gain insights into the effects of withdraw in neonates as well as the future hardships infants diagnosed with neonatal abstinence syndrome may face.

CHAPTER 4

OPIOID WITHDRAWAL BEHAVIOR IN SPINY MICE: A NOVEL PRECLINICAL MODEL OF NEONATAL OPIOID WITHDRAWAL SYNDROME (NOWS)

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ABSTRACT

As the opioid epidemic continues to grow, opioid use among pregnant women is increasing significantly. This has led to a steady rise in the number of infants born with neonatal opioid withdrawal syndrome (NOWS). Although short-term withdrawal symptoms associated with NOWS are well characterized, there are many gaps in our understanding of the short and long-term effects of prenatal opioid exposure. Current animal models of NOWS are limited by shortened gestational periods, large litter sizes, and primary organogenesis occurring after birth. This often leads to postnatal treatment to mimic drug exposure during third-trimester development. Using the unique rodent species *Acomys cahirinus*, more commonly known as spiny mice, which have an extended 40-day gestation period, small litter sizes, and increased in utero organogenesis we aim to study the short-term effects of prenatal morphine exposure by assessing withdrawal behavior. To model maternal opioid use, dams were treated daily with morphine (10 and 30 mg/kg S.C.) beginning on gestation day 19 until the day of birth; this resulted in a cumulative exposure of 19-21 days. Withdrawal behaviors for each pup were recorded daily between postnatal days 0 – 7 (PND 0-7). Our study found that prenatal morphine exposure in spiny mice led to an increase in withdrawal behavior throughout the early postnatal period and validated the use of this species as a novel pre-clinical model of NOWS. We are hopeful this rodent model will further our understanding of the short and long-term consequences of prenatal opioid exposure on neurodevelopment and behavior.

Keyword: opioids, morphine, addiction, neonatal, abstinence, withdrawal

INTRODUCTION

A critical consequence of opioid use disorder during pregnancy is the increase in the diagnosis and treatment of neonatal opioid withdrawal syndrome (NOWS). NOWS is a constellation of withdrawal symptoms experienced by infants shortly after birth due to the abrupt cessation of trans-placental drug exposure from mother to infant. Symptoms associated with NOWS typically affect the central and autonomic nervous systems as well as the gastrointestinal system. Common symptoms include tremors, irritability, excessive crying, poor feeding, sleep disturbances, increased muscle tone, fever, temperature instability, diarrhea, and in extreme cases seizures (Kocherlakota, 2014). Although several drugs have been implicated with withdrawal symptoms in neonates, it is most commonly associated with opioid use (Sutter, Leeman, & Hsi, 2014). In the United States (US) the rate of NOWS increased five-fold between 2004-2014; increasing from 1.6 to 8.8 cases per 1,000 births (Patrick et al., 2012; Winkelman, Villapiano, Kozhimannil, Davis, & Patrick, 2018). The rate of NOWS in some US states remains high, for example, in the state of West Virginia where there were 56.2 per 1,000 babies born with NOWS between 2016 – 2017. Similarly, states such as Maine (31.4 per 1,000 births) and Kentucky (23.9 per 1,000 births) also had the highest rates of NOWS. In fact, in 2017 when the national rate of NOWS births was 7.3, a total of nine US states had greater than 10 per 1,000 births that were diagnosed with NOWS, highlighting that maternal opioid abuse disorder and NOWS remains prevalent in the US (Umer et al., 2018). Additionally, the average national hospital costs associated with NOWS are over nine times (~$9,500 per baby) greater than that compared to the cost associated with non-NOWS babies (~$1,100 per baby) (Agency for Healthcare Research and Quality, 2020). Although the short-term withdrawal symptoms of NOWS are well

characterized and understood, there are still gaps in our understanding of the short and long-term effects on brain development (Boggess & Risher, 2020).

Current rodent models used to study prenatal opioid exposure have limited clinical translatability due to short gestational periods, large litter sizes, and primary organogenesis occurring postnatally. Due to the immature brain development of traditional rodent models at birth, preclinical models of prenatal opioid exposure have been developed using postnatal treatments to mimic third-trimester exposure as seen in humans (Dobbing & Sands, 1979). Unfortunately, these rodent models remove the importance of transplacental exposure of opioids from the mother during gestation limiting there clinical translatability (Ross et al., 2014). Also, large litter sizes observed in traditionally used rodent models fail to mimic human pregnancy. A rodent such as the C57BL/6 mouse species undergo significant postnatal development that limits their use to better our understanding of early, short-term effects on NOWS as seen in humans. Guinea pigs have been suggested to be a more suitable model for prenatal opioid exposure due to their lengthened gestation, small litter sizes, and precocial pups (Dobbing & Sands, 1970). Additionally, the placental structure and the metabolism of morphine is similar among guinea pigs and humans (Carter et al., 2006; Lawrence et al., 1992; Morrison et al., 2018; Murphey & Olsen, 1993). However, growth and development of the brain occurs much earlier in gestation in guinea pigs compared to humans (Dobbing & Sands, 1979). Therefore, in utero brain development still limits the translatability of this species as a model for prenatal opioid exposure for the purposes of understanding neurological and behavioral consequences. Collectively, these limitations lend to the need for a more translatable preclinical model of NOWS. We believe that spiny mice (*Acomys cahirinus*) possess several unique biological characteristics that differentiate

them from other rodents, and thus would better our understanding of NOWS to improve its treatment (early in withdrawal) and the long terms effects NOWS could have on these babies.

Spiny mice are a desert rodent species found across Africa, the Middle East, and Southern Asia that unlike their cousins, possess a menstrual cycle (Bellofiore et al., 2016; Haughton et al., 2016). In recent years, spiny mice have been highlighted as a highly translatable model to investigate neuroprotective interventions against perinatal injury and have been proposed as an ideal rodent model to study in utero development (Ellery et al., 2015; Ireland et al., 2011; Ireland et al., 2008). Together, with their ability to menstruate, spiny mice have longer gestational periods (~38-40 days), significant in utero primary organogenesis, small litter sizes (~2 -3 pups), and pups that are precocial (Dickinson & Walker, 2007). Due to their lengthened gestation, spiny mice organs undergo substantial development in utero. For example, the liver, kidney, lung, and brain are functionally mature at birth (Brunjes, 1989; Brunjes, 1985; Brunjes, Korol, & Stern, 1989b; Dickinson, Walker, Cullen-McEwen, Wintour, & Moritz, 2005b; Lamers et al., 1985; Oosterhuis et al., 1984). At 30 days of gestation, the cortical and limbic brain structures are developed to the equivalent of 24-26 weeks' gestation in the human fetus (Clancy, Darlington, & Finlay, 2001). Additionally, brain development in spiny mice has been shown to more closely resemble human development patterns. Studies have found that spiny mice experience a growth spurt in brain development around the time of birth which is sooner than other rodents like the rat and mouse, but later than the guinea pig, and is more comparable to human brain development (Quinn et al., 2016b). The small litter sizes in spiny mice allow us to study the effects of prenatal opioid exposure while minimizing the variables presented by larger litter sizes found in other rodents (Dickinson & Walker, 2007). Spiny mice are a precocial species, at birth, their bodies are covered with hair, open eyes, ears and are capable of

locomotion, and self-feeding on the second day of life (Brunjes, 1990; D'Udine et al., 1980). Due to their unique developmental characteristics, spiny mice provide a translational, preclinical model to study the effects of prenatal opioid exposure and spontaneous withdrawal symptoms associated with NOWS. Herein we evaluated the consequences of prenatal morphine exposure on withdrawal behavioral alterations analogous to those of NOWS in spiny mice.

METHODS

Breeding

All spiny mice used in this study were obtained from our in-house breeding colony maintained at Purdue University, Fort Wayne, IN. Experiments were conducted per the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Purdue Institutional Animal Care and Use Committee at Purdue University (protocol #1712001654). Spiny mice were bred male: female (1:1) starting between 9 - 12 weeks of age. Spiny mice do not produce a vaginal plug after mating, therefore the date of birth for the first litter was used to determine gestational age (days) of experimental pups. Gestational age was determined from the time of post-partum conception (i.e. mating at 24 h after delivery of a previous litter), as previously described (Dickinson et al., 2005b). A timeline of the experiments is shown in Fig. 4.1.

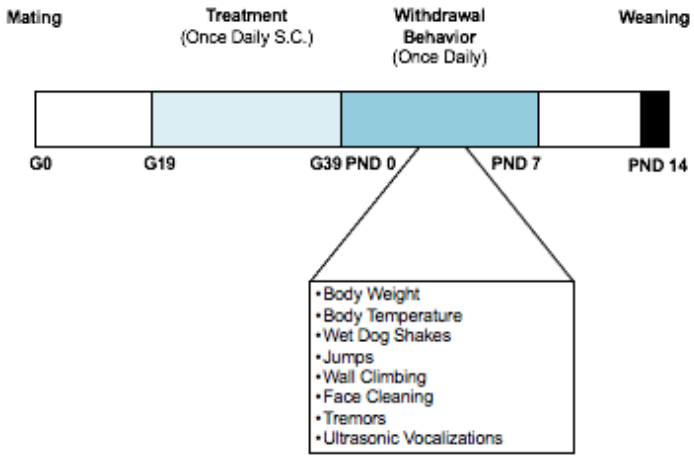

Fig. 4.1 Experimental Timeline
Saline and morphine (10 mg/kg and 30 mg/kg) was administered once-daily (S.C.) beginning on gestational day 19. During daily injections, mice were checked for general health, body weight and body temperature were recorded. Withdrawal behaviors were measured between PND 0 and PND 7 once-daily. Behavioral testing was performed in the morning by the same experimenter and data was analyzed by a blinded experimenter. All pups remained in the same cage as their mother's until weaning at PND 14, and were provided food and water *ad libitum*.

Morphine treatment

On gestational day (G) 19, dams were separated from their male partner and remained isolated throughout treatment. Dams were briefly anesthetized using 2% isoflurane and treated (S.C.) once-daily (between 09:00 – 10:00 h) with either saline (N = 6) or morphine sulfate (Spectrum Chemical, M1167) at two different concentrations, 10 and 30 mg/kg (N = 9 / dose). Morphine dosage was based on previous studies of prenatal morphine exposure in rats and mice (Mithbaokar, Fiorito, Morte, Maharajan, & Costagliola, 2016; Slamberova, Bar, et al., 2003; Slamberova, Rimanoczy, et al., 2003; Slamberova, Rimanoczy, Cao, et al., 2005). Upon birth, all pups (total N's: saline = 17; 10 mg/kg morphine = 24; and 30 mg/kg morphine = 21. See Table 4.2) remained with the dam until day of weaning at postnatal day (PND) 14. All mice were

maintained on a 12h light / dark cycle (lights on at 06:00 h) in a temperature (24 - 26 °C) and humidity (40 - 70%) controlled environment. Food and water were available *ad libitum*.

Maternal measurements

Dams were weighed daily beginning on G19 to determine accurate volume adjustments for daily treatments and to confirm continuous pregnancy. The body temperature of dams was also recorded daily during gestation. Following parturition, body weight and temperature of all dams were also recorded once-daily during the first seven PND's to monitor possible symptoms associated with opioid withdrawal following treatment cessation.

Litter characteristics

Cages were checked each morning between 08:00 – 09:00 h for new litters. The day of parturition was designated as PND 0 and pups were examined to determine sex using anogenital distance. On the same day, litter size, weight, and body temperature of the pups were assessed. If any deceased pups were found in the cage it was recorded before being removed; sex was recorded as unknown if the remains of the pup did not allow for an accurate sex classification (Table 4.2). Beginning on PND 0, each pup was evaluated for NOWS using the following behavioral assays.

Behavioral procedures

All behavioral testing was conducted daily between 08:00 – 10:00 h on PND 0 – 7. Before testing began, mice were placed in the testing room for a minimum of 30 minutes to acclimate. The testing room temperature was maintained at 24 °C (75 °F) and the humidity was

set between 40 - 70%. Spiny mice were tested and recorded to allow for more accurate post-testing observations (blinded) and data collection. The behavioral assays were performed in the order in which they are described below.

Spontaneous withdrawal

Symptoms of opioid withdrawal were assessed with each pup between PND 0 - 7. Each day, pups were removed from their home cage and placed in a clear plastic observation chamber. Before each test, the observation chamber was cleaned with 70% ethanol followed by water and dried to remove any olfactory cues. On each day, the body temperature (°C) was measured immediately following removal from the home cage using an infrared thermometer, and the body weight (grams) was measured with a digital scale designed to be used with small animals (Redmon, Peru, IN, USA). Pups were allowed to freely explore the observation chamber for three minutes and behaviors were observed, recorded, and then scored using the open-source Behavioral Observational Research Interactive Software (BORIS; http://www.boris.unito.it) by experimenters blinded to the treatment groups. Withdrawal behaviors that were scored during the three-minute observation period included wet-dog shakes, face cleaning, wall climbing, jumping, and tremors (See Table 4.1 for definitions of withdrawal behaviors) (G A Barr et al., 2011; Richardson et al., 2006).

Behavior	Description
Wet dog shakes	Rapid shaking of the whole body
Jumps	Sudden leaping such that all four paws are off the bottom of the chamber
Face cleaning	The continuous movement of paws towards the face
Wall climbing	Putting both forepaws on the wall of the observation chamber; typically, with movement
Ultrasonic vocalizations	Vocalizations in the ultrasonic range (20-128 kHz)
Tremor	Spontaneous shaking or kicking of the hind limbs with full-body movement; Shaking, twitching, curling, or sweeping movement of the tail

Table 4.1. Neonatal opioid withdrawal syndrome (NOWS) behavior

Ultrasonic vocalizations (USV's)

Immediately following the completion of withdrawal behavior observations, pups were then placed into a glass container housed inside a sound-attenuating box. Before each test, this container was cleaned with 70% ethanol followed by water and dried to remove any olfactory cues. Ultrasonic vocalization (USV) emissions were recorded by the Echo Meter Touch® bat detector (Wild Life Acoustics, Maynard, MA) attached to an iPhone 8 (Apple, Cupertino, CA) that was mounted inside the roof of the sound attenuating box. USV's were recorded daily for each pup for two minutes from PND 0 - 7. Immediately following completion of recordings, pups were placed back in their home cage. For acoustical analysis, recordings were transferred to RavenPro® software (Cornell Laboratory of Ornithology, Ithaca, NY) and USV's occurring within the range of 20-125 kHz were quantified. All behavioral assays were performed in a quite behavioral suite and completed within ten minutes to minimize the stress on pups of being separated from their mother.

Data Analysis

All data are presented as mean ± SEM. Maternal body weight and body temperature were assessed using two-way repeated-measures ANOVA with Tukey's post hoc test, and treatment and PND's as factors. Length of exposure and litter size were assessed using one-way ANOVA with Tukey's post hoc test. The pup death rate was assessed using an ordinary one-way ANOVA with Tukey's post hoc test. Spontaneous withdrawal behaviors were assessed using two-way repeated-measures ANOVA with Tukey's post hoc test, with treatment and PND's as factors. Sex differences within treatment groups were analyzed using two-way repeated-measures ANOVA with Sidak's post hoc test with sex and PND's as factors. All data, including Area

under the curve (AUC ± SEM) were analyzed with GraphPad Prism version 8.0 (San Diego, CA, USA) and differences were considered significant for $P < 0.05$.

RESULTS

General conditions of dams

Body weight and body temperatures were measured daily for each dam between G19 and G40 (Fig. 4.2a-c). Morphine treated dams had a slower rate of weight gain throughout this period compared to the saline-treated dams (Fig. 4.2a). Dams from the 30 mg/kg morphine treatment group had significantly higher body temperatures on the 2nd day of treatment compared to the 10 mg/kg morphine group (31.19 ± 0.16 vs. 30.11 ± 0.32). On the 6th day of treatment, dams from the 30 mg/kg morphine group had a significantly higher body temperature compared to the saline-treated group (31.09 ± 0.22 vs. 30.10 ± 0.40) ($P < 0.05$) (Fig. 4.2c). Bodyweight were also recorded daily on PND's 0-7 (Fig. 4.2b-d). Dams from the morphine treatment groups had lower body weights compared to dams from the saline group (not significant) (Fig. 4.2b). Body temperatures on PND's were not significantly different from the saline-treated mice (Fig. 4.2d).

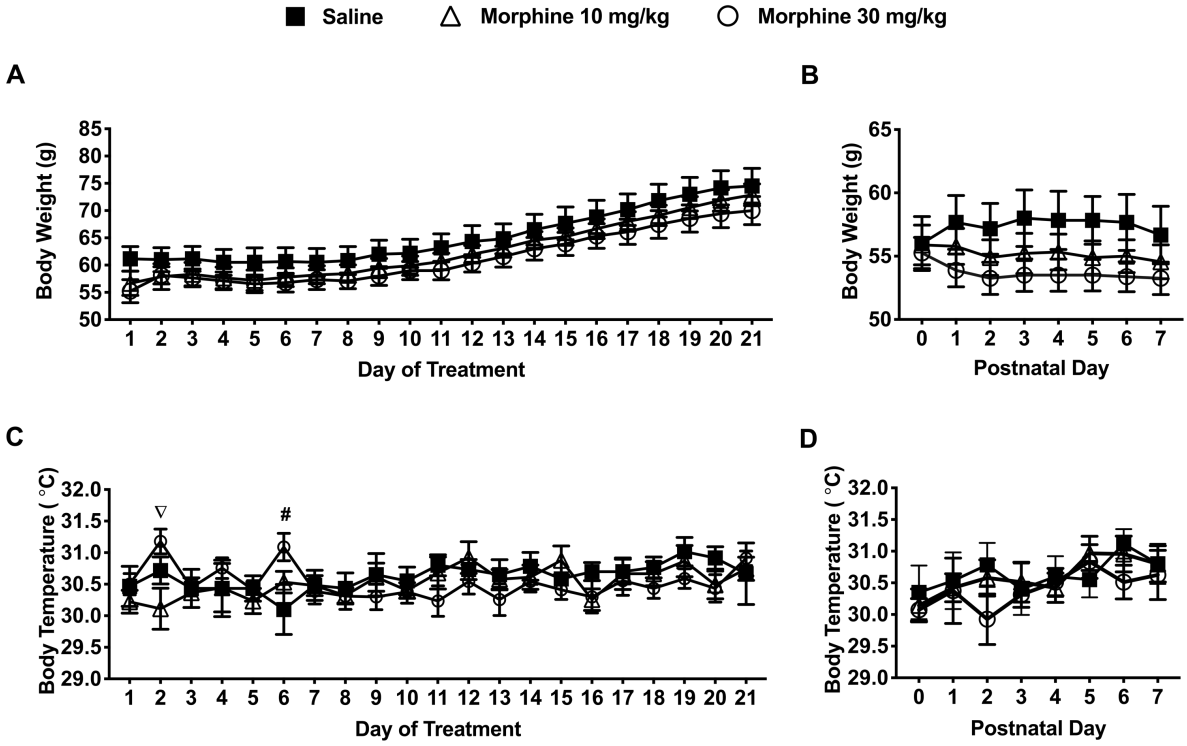

Fig. 4.2 Maternal changes during the gestational treatment period and early postpartum period (PND 0-7).

a) There was no significant difference between treatment groups on the body weights of dams during gestation or during the early postpartum period (PND 0-7) (AUC [Weight] – Saline: 1302 ± 21.12; 10 mg/kg: 1251 ± 17.38; 30mg/kg: 1225 ± 18.66). (b) During the early postpartum period (PND 0 - 7), 10mg/kg morphine (N = 9) and 30 mg/kg morphine (N = 9) treated dams had lower (non-significant) body weights compared to saline-treated controls (N =6) (AUC [Weight] – Saline: 402.5 ± 9.82; 10 mg/kg: 386.3 ± 7.95; 30mg/kg: 375.3 ± 6.64). (c) On the 2nd day of treatment, dams treated with 30mg/kg morphine (N = 9) had significantly higher body temperatures compared to 10 mg/kg morphine (N = 9) treated dams. On the 6th day of treatment, spiny mice treated with 30mg/kg morphine (N = 9) had significantly higher body temperatures than compared to saline-treated controls (N = 6) (AUC [Temperature] – Saline: 612.5 ± 1.99; 10 mg/kg: 610.6 ± 2.11; 30mg/kg: 610.3 ± 1.85). (d) During the early postpartum period (PND 0-7) there was no significant difference in body temperatures between treatment groups (AUC [Temperature] – Saline: 214.6 ± 1.61; 10 mg/kg: 214.3 ± 1.52; 30mg/kg: 212.8 ± 1.73). Data are presented as means totals (±SEM) during an approximate 21-day treatment/gestation period and a PND 0 – 7 period. An pound symbol (# = saline vs. 30 mg/kg morphine) or nabla symbol (∇ =10 mg/kg morphine vs. 30 mg/kg morphine) are used to indicate a significant difference between groups, $P < 0.05$.

Spiny mice pup characteristics

There was no significant difference in the length of exposure (days) between the morphine (10 and 30 mg/kg) and saline-exposed groups (21.33 ± 0.17 and 21.44 ± 0.29 vs. saline: 20.67 ± 0.49 respectively). The average litter size was smaller in the morphine (10 and 30 mg/kg) exposed groups compared to the saline-exposed group (2.67 ± 0.29 and 2.44 ± 0.29 vs. saline: 2.83 ± 0.17 respectively). There was a higher percentage of dead pups (26.09 ± 9.36%) from the 30 mg/kg morphine exposed group compared to the pups from the 10 mg/kg morphine and saline exposed groups ($P < 0.05$) (See Table 4.2). In terms of sex, more female pups died (44.44 ± 17.57%) compared to males within the 30 mg/kg morphine exposed group ($P < 0.05$) (Table 4.2).

Treatment	Saline	10 mg/kg	30 mg/kg	
Number of dams	6	9	9	
Number of litters	6	9	9	
Number of pups	17	24	23	
Number male	8	12	12	
Number female	9	12	9	
Number unknown	0	0	2	
Length of exposure (days)	20.67 ± 0.49	21.33 ± 0.17	21.44 ± 0.29	NS
Mean litter size	2.83 ± 0.17	2.67 ± 0.29	2.44 ± 0.29	NS
Deaths				
Male	0	0	0	
Female	0	0	4	
Unknown	0	0	2	

Table 4.2. Maternal and litter characteristics

The Measure of Spontaneous Opioid Withdrawal in Pups

Body temperature

Withdrawal from prenatal morphine exposure increased body temperature in spiny mice pups during the early postnatal period (Fig. 4.3a-b). On PND's 2 – 7, male mice from both morphine (10 and 30 mg/kg) exposed groups had significantly higher body temperatures compared to saline ($P < 0.05$) (Fig. 4.3a). Similarly, on PND's 0 - 7 female mice from both morphine (10 and 30 mg/kg) exposed groups also had significantly higher average body temperatures compared to saline ($P < 0.05$) (Fig. 4.3b). No significant difference in body temperatures was found between sexes among all exposure groups and PND's.

Body weight

Withdrawal from prenatal morphine exposure resulted in a decrease in body weight in spiny mice pups during the early postnatal period (Fig. 4.3c-d). On PND's 4, 5, 6, and 7, male mice exposed to 10 mg/kg morphine had significantly lower body weights compared to the saline exposed males (8.50 ± 0.20; 9.75 ± 0.28; 10.58 ± 0.23 and 11.50 ± 0.26 vs. saline: 9.63 ± 0.26; 10.63 ± 0.26; 12.00 ± 0.19 and 12.88 ± 0.30 respectively) ($P < 0.05$) (Fig. 4.3c). Similarly, on PND's 1, 3, 4, 6 and 7, male mice exposed to 30 mg/kg morphine group also had significantly lower body weights compared to the saline exposed males (5.92 ± 0.08; 7.75 ± 0.13; 8.75 ± 0.22; 11.08 ± 0.26 and 12.08 ± 0.26 vs. saline: 6.75 ± 0.16; 8.63 ± 0.18; 9.63 ± 0.26; 12.00 ± 0.19 and 12.88 ± 0.30 respectively) ($P < 0.05$) (Fig. 4.3c). Although a lower body weight was observed in female mice exposed to morphine, no significance was observed when compared to the saline exposed females (Fig. 4.3d). No significant difference in body weight was found between sexes across all exposure groups and PND's.

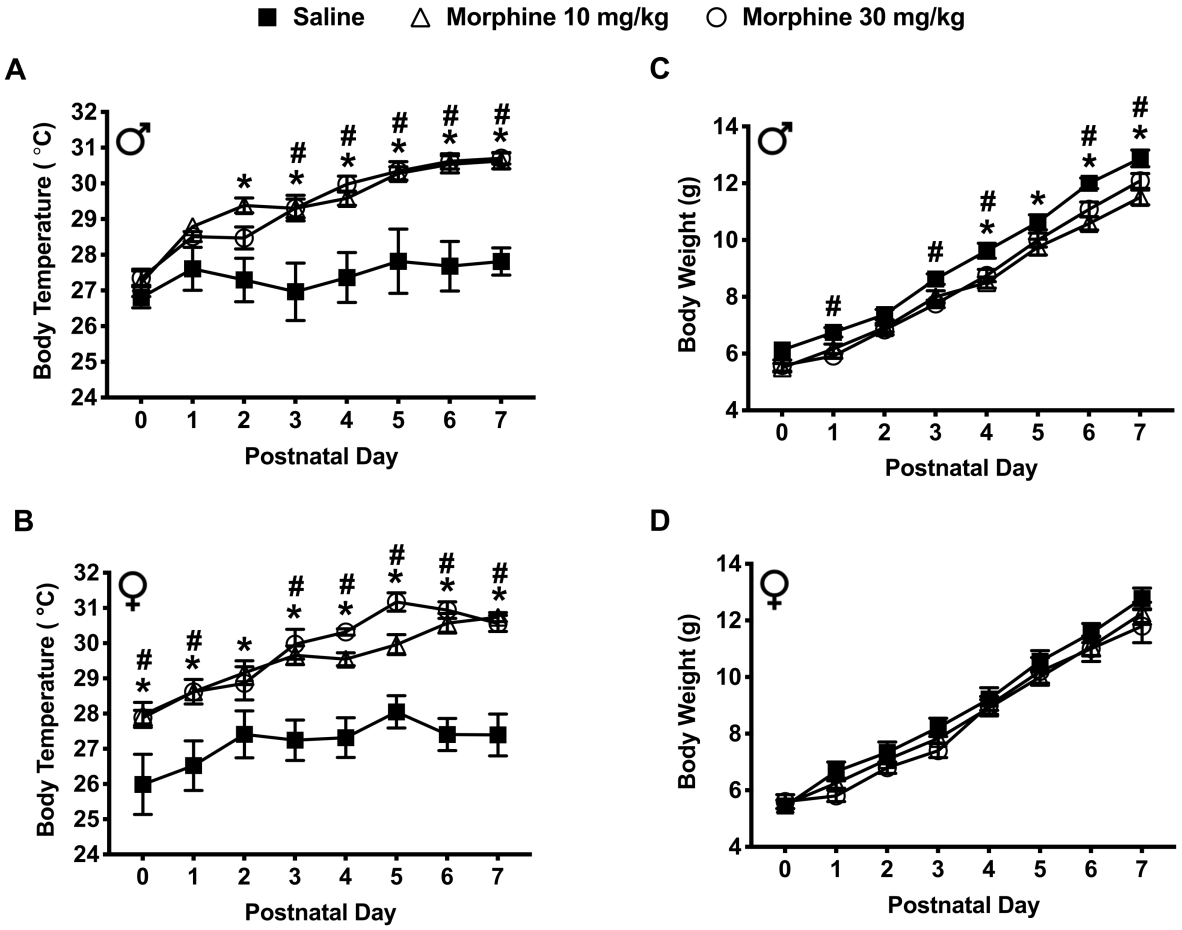

Fig. 4.3 Spontaneous withdrawal following prenatal morphine (10 and 30 mg/kg) exposure affected the body temperature and body weight of spiny mice pups.
(a) Male pups exposed to 10mg/kg morphine (N = 12) and 30mg/kg morphine (N=12) had significantly higher body temperatures compared to the saline-exposed controls (N = 8) (AUC [Temperature] – Saline: 192.0 ± 3.63; 10 mg/kg: 206.8 ± 1.52; 30mg/kg: 206.3 ± 1.63). (b) Similarly, female pups exposed to 10mg/kg morphine (N = 12) and 30 mg/kg morphine (N = 9) had significantly higher body temperatures compared to the saline-exposed controls (N = 9) (AUC [Temperature] – Saline: 190.6 ± 3.39; 10 mg/kg: 206.9 ± 1.67; 30mg/kg: 209.1 ± 1.32). (c) Male pups exposed to 10 mg/kg morphine (N=12) and 30 mg/kg morphine (N = 12) had significantly lower body weights compared to saline-exposed controls (N = 8) (AUC [Weight] – Saline: 64.50 ± 1.13; 10 mg/kg: 58.42 ± 1.36; 30mg/kg: 59.17 ± 1.30). (d) In contrast, there were no significant differences in body weight in 10 mg/kg morphine (N = 12) or 30 mg/kg morphine (N = 9) exposed female pups compared to the saline-exposed controls (AUC [Weight] – Saline: 62.67 ± 1.96; 10 mg/kg: 60.04 ± 1.57; 30mg/kg: 58.90 ± 1.48). Data are presented as means total (±SEM) during a PND 0 – 7 period. An asterisk symbol (* = saline vs. 10 mg/kg morphine) or pound symbol (# = saline vs. 30 mg/kg morphine) are used to indicate a significant difference between groups, $P < 0.05$.

Spontaneous jumps

Withdrawal from prenatal morphine exposure resulted in a significant increase in jumping behavior in spiny mice pups during the early postnatal period (Fig. 4.4a-b). On PND 6, male mice exposed to 10 mg/kg morphine made a significantly greater number of jumps compared to the saline exposed males (11.50 ± 2.69 vs. 6.50 ± 2.84) ($P < 0.05$) (Fig.4.4a). Additionally, on PND 6, males exposed to 10 mg/kg morphine made a significantly greater number of jumps compared to the morphine 30 mg/kg exposed males (11.50 ± 2.69 vs. 5.08 ± 1.43). On PND's 5, 6, 7, female mice exposed to 10 mg/kg morphine also made a significantly greater number of jumps compared to the saline exposed females (8.75 ± 2.50; 12.92 ± 2.32 and 13.75 ± 2.57 vs. 0.89 ± 0.56; 2.33 ± 0.90 and 6.33 ± 1.97) ($P < 0.05$) (Fig.4.4b). On PND's 5 and 6, female mice exposed to 10 mg/kg morphine had a significantly greater number of jumps compared to the 30 mg/kg morphine exposed females (8.75 ± 2.50; 12.92 ± 2.32 vs. 1.60 ± 0.81; 7.60 ± 0.98) ($P < 0.05$) (Fig. 4.4b). There was no significant difference in the number of jumps between sexes across all exposure groups and PND's.

Wet dog shakes

Withdrawal from prenatal morphine exposure resulted in a significant increase in wet dog shaking behavior in spiny mice pups during the early postnatal period (Fig. 4.4c-d). On PND 2, male mice exposed to 10 mg/kg morphine made a significantly greater number of wet dog shakes compared to the saline exposed males (0.92 ± 0.19 vs. 0.00 ± 0.00) ($P < 0.05$) (Fig. 4.4c). Similarly, on PND 0, 2, & 3, male mice exposed to 30 mg/kg morphine made a significantly greater number of wet dog shakes compared to males exposed to saline (1.00 ± 0.43; 1.08 ± 0.29 and 0.92 ± 0.23 vs. 0.00 ± 0.00) ($P < 0.05$) (Fig. 4.4c). On PND 1, 2, & 4, female mice exposed

to 10 mg/kg morphine made a significantly greater number of wet dog shakes compared to the saline exposed females (1.31 ± 0.37; 1.39 ± 0.31 and 0.92 ± 0.24 vs. saline: 0.11 ± 0.11; 0.00 ± 0.00 and 0.11 ± 0.11) ($P < 0.05$) (Fig. 4.4d). On PND 1, female mice exposed to 10 mg/kg morphine made a significantly greater number of wet dog shakes compared to females exposed to 30 mg/kg morphine (1.31 ± 0.37 vs. 0.20 ± 0.20) ($P < 0.05$) (Fig. 4.4d). There was no significant difference in the number of wet dogs shakes between sexes across all exposure groups and PND's.

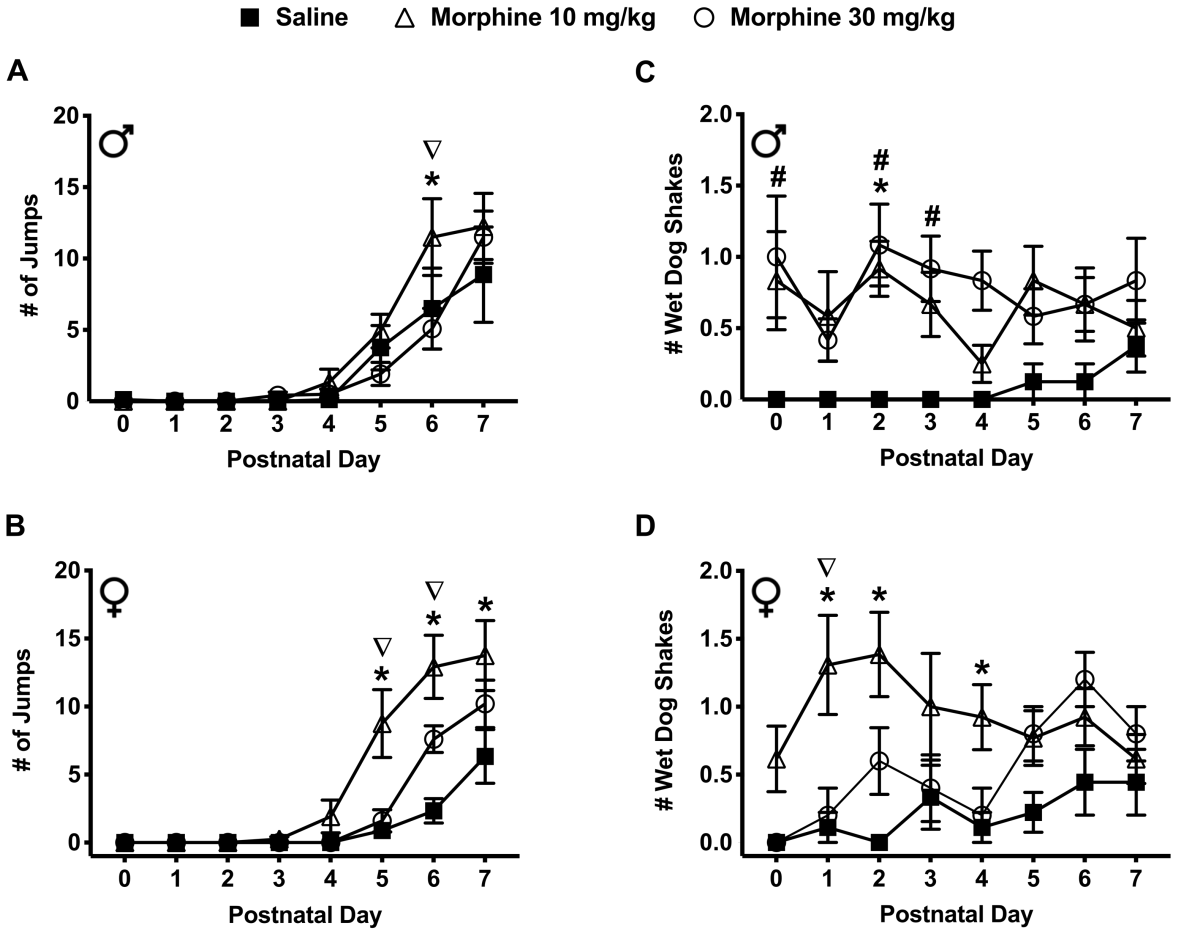

Fig. 4.4 Spontaneous withdrawal following prenatal morphine (10 and 30 mg/kg) exposure affected jumping and wet dog shaking behaviors in spiny mice pups.
(a) Male pups on PND-6 exposed to 10 mg/kg morphine (N = 12) made a significantly greater number of jumps compared to the saline-exposed controls (N = 8) and the 30 mg/kg morphine exposed group (N = 12) (AUC [#Jumps] – Saline: 14.88 ± 8.01; 10 mg/kg: 23.88 ± 8.53; 30mg/kg: 13.67 ± 5.24). (b) Similarly, female pups on PND-5, 6, and 7 from the 10 mg/kg morphine exposed group (N = 12) made a significantly greater number of jumps compared to the saline-exposed controls (N = 9) and 30 mg/kg morphine exposed group (N = 9) on PND-5,6 (AUC [#Jumps] – Saline: 6.39 ± 3.72; 10 mg/kg: 30.71 ± 9.93; 30mg/kg: 14.30 ± 2.80). (c) Male pups on PND-0, 2, and 3 from the 30 mg/kg morphine exposed group (N = 12) made a significantly greater number of wet dog shakes compared to the saline-exposed controls (N = 8). On PND 2, males from 10 mg/kg morphine exposed group (N=12) made a significantly greater number of wet dog shakes compared to saline-exposed controls (N = 8) (AUC [#Shakes] – Saline: 0.44 ± 0.44; 10 mg/kg: 4.58 ± 1.56; 30mg/kg: 5.42 ± 1.56). (d) Female pups on PND-1, 2, and 4 from the 10 mg/kg morphine exposed group (N = 12) made a significantly greater number of wet dog shakes compared to the saline-exposed controls (N = 9) and 30 mg/kg morphine treated mice (N = 9) on PND-2 (AUC [#Shakes] – Saline: 1.44 ± 0.92; 10 mg/kg: 6.92 ± 1.93; 30mg/kg: 3.80 ± 0.87). Data are presented as means totals (±SEM) during the PND 0 – 7 period. An asterisk symbol (* = saline vs. 10 mg/kg morphine) or pound symbol (# =

saline vs. 30 mg/kg morphine) or nabla symbol (∇ =10 mg/kg morphine vs. 30 mg/kg morphine) are used to indicate a significant difference between groups, $P < 0.05$.

Wall climbing

Withdrawal from prenatal morphine exposure increased wall climbing behavior in spiny mice pups during the early postnatal period (Fig. 4.5a-b). On PND 4, male mice exposed to 10 mg/kg morphine made a significantly greater number of wall climbs compared to the saline exposed males (12.92 ± 1.25 vs. 6.75 ± 2.25 respectively) ($P < 0.05$) (Fig. 4.5a). On PND 3, 4, & 5, female mice exposed to 10 mg/kg morphine also made a significantly greater number of wall climbs compared to the saline exposed females (8.92 ± 1.21; 13.67 ± 1.09 and 17.92 ± 1.66 vs. 4.00 ± 1.66; 8.89 ± 1.55 and 11.89 ± 2.18) ($P < 0.05$) (Fig. 4.5b). On PND's 5 & 6, female mice exposed to 30 mg/kg morphine made a significantly greater number of wall climbs compared to the saline exposed females (19.00 ± 1.92; 22.40 ± 2.62 vs. 11.89 ± 2.18; 16.22 ± 1.99 respectively) ($P < 0.05$) (Fig. 4.5b). There was no significant difference in the number of walls climbs between sexes across all exposure groups and PND's**.**

Face cleaning

Withdrawal from prenatal morphine exposure increased face cleaning behavior in spiny mice pups during the early postnatal period (Fig. 4.5c-d). On PND 1, male mice exposed to 30 mg/kg morphine made a significantly greater number of face cleanings compared to the saline exposed males (2.08 ± 0.56 vs. 0.38 ± 0.18 respectively) ($P < 0.05$) (Fig. 4.5c). Additionally, on PND 1, male mice exposed to 30 mg/kg morphine made a significantly greater number of face cleanings compared to males exposed to 10 mg/kg morphine (2.08 ± 0.56 vs 1.00 ± 0.30 respectively) ($P < 0.05$) (Fig. 4.5c). On PND 1, female mice exposed to 10 mg/kg morphine also

made a significantly greater number of face cleanings compared to the saline exposed (2.75 ± 0.57 vs. 0.67 ± 0.24) and 30mg/kg morphine exposed females (2.75 ± 0.57 vs. 0.80 ± 0.58) ($P < 0.05$) (Fig. 4.5d). Additionally, on PND 1, female mice exposed to 10 mg/kg morphine made a significantly greater number of face cleanings compared to male mice exposed to 10 mg/kg morphine (2.75 ± 0.57 vs. 1.00 ± 0.30 respectively) ($P < 0.05$) (Fig. 4.5d).

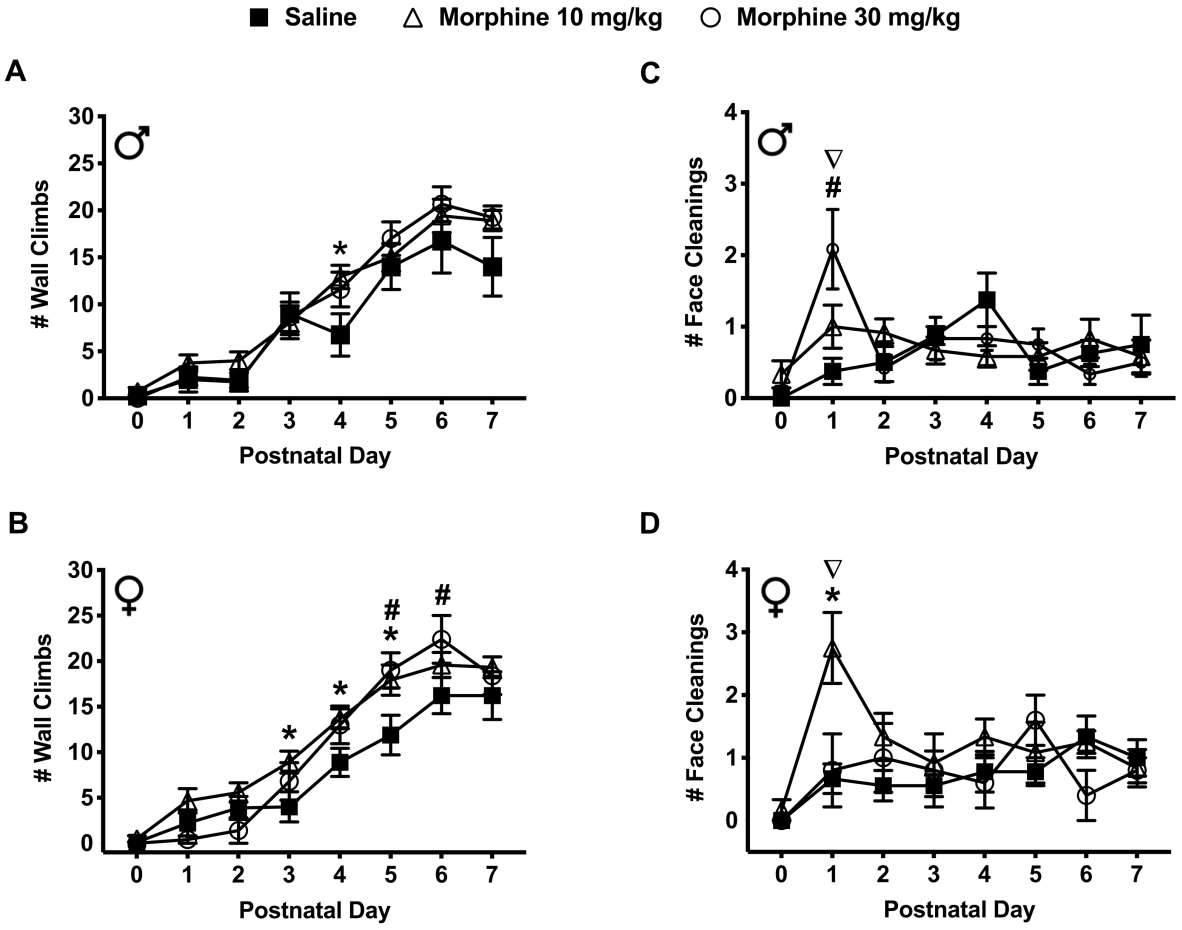

Fig. 4.5 Spontaneous withdrawal following prenatal morphine (10 and 30 mg/kg) exposure affected wall climbing and face cleaning behavior in spiny mice pups.
(a) Male pups on PND-4 from the 10 mg/kg morphine exposed group (N = 12) made a significantly greater number of wall climbs compared to the saline-exposed controls (N = 8) (AUC [#Climbs] – Saline: 57.38 ± 11.87; 10 mg/kg: 73.00 ± 8.58; 30mg/kg: 71.71 ± 9.35). (b) Similarly, female pups on PND-3, 4, 5 from the 10 mg/kg morphine exposed group (N = 12) and PND-5, 6 pups from the 30 mg/kg morphine exposed group (N = 9) made a significantly greater number of wall climbs compared to the saline-exposed controls (N = 9) (AUC [#Climbs] – Saline: 55.28 ± 9.69; 10 mg/kg: 80.25 ± 8.11; 30mg/kg: 72.20 ± 7.64). (c) Male pups on PND-1 from the 30 mg/kg morphine exposed group (N = 12) made a significantly greater number of face cleanings compared to the saline-exposed controls (N = 8) and 10 mg/kg morphine exposed group (N = 12) (AUC [#Cleans] – Saline: 4.50 ± 1.29; 10 mg/kg: 5.04 ± 1.43; 30mg/kg: 5.54 ± 1.82). (d) Female pups on PND-1 from the 10 mg/kg morphine exposed group (N = 12) made a significantly greater number of face cleanings compared to saline-exposed controls (N = 9) and the 30 mg/kg morphine exposed group (N = 9) (AUC [#Cleans] – Saline: 5.17 ± 1.43; 10 mg/kg: 9.17 ± 2.33; 30mg/kg: 5.60 ± 1.92). Data are presented as means totals (±SEM) during the PND 0 – 7 period. An asterisk symbol (* = saline vs. 10 mg/kg morphine) or pound symbol (# = saline vs. 30 mg/kg morphine) or nabla symbol (∇ =10 mg/kg morphine vs. 30 mg/kg morphine) are used to indicate a significant difference between groups, $P < 0.05$.

Tremors

Withdrawal from prenatal morphine exposure increased tremor behavior in spiny mice pups during the early postnatal period (Fig. 4.6a-b). On PND 0, male mice exposed to morphine (10 and 30 mg/kg) experienced a significantly greater number of tremors compared to the saline exposed males (3.83 ± 1.14 and 6.08 ± 1.39 vs. 0.00 ± 0.00, respectively) ($P < 0.05$) (Fig. 4.6a). In addition, males exposed to 30 mg/kg morphine experienced a significantly greater number of tremors compared to males exposed to 10 mg/kg morphine (6.08 ± 1.39 vs. 3.83 ± 1.14) ($P < 0.05$) (Fig. 4.6a). On PND 0, female mice exposed to 10 mg/kg morphine also experienced a significantly greater number of tremors compared to the saline exposed mice (7.42 ± 1.86 vs. 0.00 ± 0.00) ($P < 0.05$) (Fig. 4.6b). Additionally, females exposed to 10 mg/kg morphine experienced a significantly greater number of tremors compared to the 30 mg/kg morphine exposed females (7.42 ± 1.86 vs. 1.40 ± 1.17) ($P < 0.05$) (Fig. 4.6b). Interestingly, the number of tremors observed in the morphine exposed groups was significantly different between sexes. On PND 0, among pups exposed to 10 mg/kg morphine, females experienced a significantly greater number of tremors compared to males (7.42 ± 1.86 vs. 3.83 ± 1.14) ($P < 0.05$). In contrast, on PND 0, among pups exposed to 30 mg/kg morphine, male mice experienced a significantly greater number of tremors compared to female mice (6.08 ± 1.39 vs. 1.40 ± 1.17) ($P < 0.05$) (Fig. 4.6a-b).

Ultrasonic vocalizations (USV's)

Withdrawal from prenatal morphine exposure resulted in a significant decrease in the number of ultrasonic vocalizations (USV's) in spiny mice pups during the early postnatal period (Fig. 4.6c-d). On PND's 2, 3 & 4, male mice exposed to 10 mg/kg morphine emitted

significantly fewer number of USV's compared to the saline exposed mice (70.17 ± 11.70; 72.00 ± 6.59 and 66.75 ± 7.64 vs. 123.63 ± 12.74; 118.13 ± 10.31 and 112.63 ± 10.72) ($P < 0.05$) (Fig. 4.6c). Similarly, on PND's 2, 4 & 5, male exposed to 30 mg/kg morphine emitted significantly fewer number of USV's compared to mice from the saline exposed group (84.33 ± 10.25; 73.17 ± 11.66 and 71.75 ± 6.62 vs. 123.63 ± 12.74; 112.63 ± 10.72 and 103.25 ± 5.66) ($P < 0.05$) (Fig. 4.6c).

Similar to the males, on PND's 2, 3 & 4, female mice exposed to 10 mg/kg morphine emitted a significantly fewer number of USV's compared to mice exposed to saline (78.08 ± 9.70; 89.17 ± 11.06 and 71.00 ± 6.72 vs. 123.67 ± 10.47; 120.67 ± 10.35 and 117.00 ± 8.30) ($P < 0.05$) (Fig. 4.6d). On PND's 1, 2, 3 & 6, female mice exposed to 30 mg/kg morphine emitted significantly fewer USV's compared to the mice from the saline exposed group (22.67 ± 14.54; 30.67 ± 9.76; 53.17 ± 9.60 and 21.17 ± 7.85 vs. 113.67 ± 6.67; 123.67 ± 10.47; 120.67 ± 10.35 and 73.56 ± 5.38) ($P < 0.05$) (Fig. 4.6d). On PND's 1, 2, 3 & 6, female mice exposed to 10 mg/kg morphine emitted significantly greater number of USV's compared to female exposed to 30 mg/kg morphine (96.33 ± 13.12; 78.08 ± 9.70; 89.17 ± 11.06 and 58.58 ± 9.31 vs. 30 mg/kg Morphine : 22.67 ± 14.54; 30.67 ± 9.76; 53.17 ± 9.60 and 21.17 ± 7.85) ($P < 0.05$) (Fig. 4.6d). The number of USV's emitted was also significantly different between sexes. On PND's 1, 2, 6 & 7 female mice emitted significantly fewer USV's compared to male mice exposed to 30 mg/kg morphine (22.67 ± 14.54; 30.67 ± 9.76; 21.17 ± 7.85; 15.00 ± 9.62 vs. 77.83 ± 8.34; 84.33 ± 10.25; 67.08 ± 8.53 and 56.42 ± 8.98 respectively) ($P < 0.05$) (Fig. 4.6c-d).

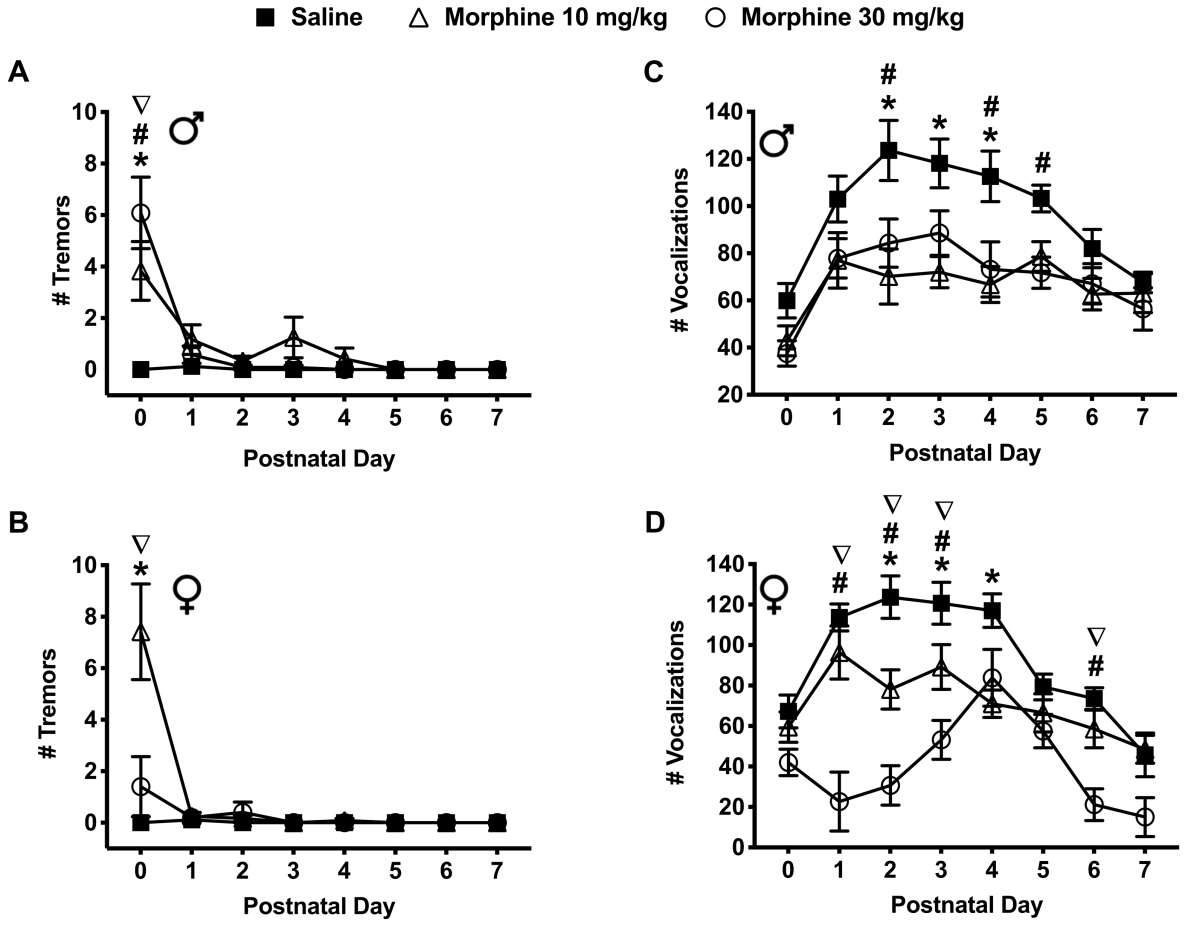

Fig. 4.6 Spontaneous withdrawal following prenatal morphine (10 and 30 mg/kg) exposure affected tremor and vocalization behaviors in spiny mice pups.
(a) Male pups on PND-1 from the 10 mg/kg morphine (N =12) and 30 mg/kg morphine (N=12) exposed groups experienced a significantly greater number of tremors compared to the saline-exposed controls (N = 8). Pups from the 30 mg/kg morphine exposed group (N = 12) experienced a significantly greater number of tremors compared to the 10 mg/kg morphine exposed group (N=12) (AUC [#Tremors] – Saline: 0.13 ± 0.25; 10 mg/kg: 5.08 ± 3.30; 30mg/kg: 3.79 ± 2.56). (b) Similarly, female pups on PND-1 from the 10 mg/kg morphine exposed group (N = 12) experienced a significantly greater number of tremors compared to the saline-exposed controls (N = 9) and 30 mg/kg morphine exposed group (N = 9) (AUC [#Tremors] – Saline: 0.11 ± 0.24; 10 mg/kg: 4.21 ± 3.25; 30mg/kg: 1.30 ± 1.48). (c) Male pups on PND-2, 3, and 4 from the 10 mg/kg morphine (N = 12) and on PND-2,4, and 5 from the 30 mg/kg morphine (N=12) exposed groups emitted significantly fewer ultrasonic vocalizations compared to the saline-exposed controls (N = 8) (AUC [#Vocalizations] – Saline: 706.4 ± 49.48; 10 mg/kg: 480.2 ± 55.63; 30mg/kg: 509.7 ± 58.52). (d) Similarly, female pups on PND-2, 3, and 4 from the 10 mg/kg morphine exposed (N = 12) and on PND-1, 2, 3, and 6 from the 30 mg/kg morphine exposed group (N=9) emitted significantly fewer ultrasonic vocalizations compared to the saline-exposed controls (N=9). Additionally, on PND-1, 2, 3, 6 female pups from the 30mg/kg morphine exposed group (N = 9) emitted significantly fewer ultrasonic

vocalizations compared to the 10 mg/kg morphine exposed group (N = 12) (AUC [#Vocalizations] – Saline: 684.4 ± 46.99; 10 mg/kg: 513.7 ± 63.01; 30mg/kg: 297.5 ± 48.76). Data are presented as means totals (±SEM) during the PND 0 – 7 period. An asterisk symbol (* = saline vs. 10 mg/kg morphine) or pound symbol (# = saline vs. 30 mg/kg morphine) or nabla symbol (∇ =10 mg/kg morphine vs. 30 mg/kg morphine) are used to indicate a significant difference between groups, $P < 0.05$.

DISCUSSION

Our study is the first to examine the effects of prenatal morphine exposure in spiny mice. We performed physiological and behavioral tests in spiny mice pups prenatally exposed to morphine to characterize symptoms of withdrawal in this unique rodent species. The lengthened gestational period and increased in utero organogenesis of the spiny mouse allows for improved characterization and understanding of opioid withdrawal in pups following prenatal opioid exposure. Data presented in this study supported our hypothesis that spiny mouse would make an improved preclinical model of NOWS. Our novel model of NOWS relies on trans-placental exposure of morphine instead of postnatal opioid exposure, commonly used in other rodent models to mimic third-trimester exposure. Compared to other rodent species, spiny mice undergo advanced organogenesis and are precocial at birth (Brunjes, 1990). This allowed us to capture withdrawal symptoms that would otherwise not be possible in other rodent models during the early postnatal period.

Dams' body weight and temperature were monitored daily to assess any response to morphine treatment during gestation and during the first 7 days following parturition. Although differences in body weight observed during the gestation and postpartum periods were not significant, a decreased rate of weight gain among dams was measured in the morphine treated groups. Additionally, an increase in body temperature was only observed in dams from the 30 mg/kg morphine treatment group during the first 7 days of treatment. The fluctuations in body

temperature during pregnancy may have been due to dams initially adjusting to daily treatments of high dose morphine and also the possible consequence of carrying an unborn litter.

Morphine treatment of pregnant spiny mice also affected litter size. In our study, the average litter size was smaller in the morphine (10 and 30 mg/kg) treated groups compared to saline-treated groups. As mentioned above, dams from the morphine treatment groups consistently presented with lower body weights during the periods of morphine treatment and postpartum and it is unclear if the smaller litter sizes from the morphine treated dams could correlate to the reduced weight gain observed in dams. Similarly, we are uncertain if morphine administration could have influenced the spontaneous death of pups and/or fetal absorption in utero as treatment with the higher, 30 mg/kg morphine dose significantly increased the number of deceased pups. More specifically, female pups from the higher, 30 mg/kg morphine group were most affected. The cannibalism of offspring by dams within the 30 mg/kg morphine treatment group was also observed. Endogenous opioids are known to play a key role in the regulation of the neuroendocrine axis and the initiation of maternal caregiving (Bicknell, 1985; Cruz, Maiorka, Canteras, Sukikara, & Felicio, 2010; Farid, Dunlop, Tait, & Hulse, 2008; Morley, 1981; Stafisso-Sandoz, Polley, Holt, Lambert, & Kinsley, 1998). Previous studies have reported a decrease in maternal care and an increase in cannibalism following gestational opioid exposure (Chen et al., 2015; Wallin et al., 2019). Although not conclusive, it seems as though the deaths associated with morphine treatment were not perhaps directly linked to the lack of maternal care, but instead an effect of morphine on the pup's ability to survive. Careful observations of dams treated with 30 mg/kg morphine showed attempts to feed, groom, and care for their pups. However, pups that were severely affected were typically unable to feed regularly and exhibited signs of failure to thrive.

Prenatal morphine exposure did not result in all pups within a litter dying. Instead, one pup was more effected than their litter mates. Studies have shown that prenatal drug exposure can be complicated by the uterine position of pups; where one pup may have greater drug exposure than another pup in the same litter (Lipton, Robie, Ling, Weese-Mayer, & Carvey, 1998). Fortunately, with the litter size of spiny mice being smaller than other rodents, any intra-pups' variability of the symptoms associated with withdrawal was minimized. Furthermore, unequal exposure to morphine cannot explain why the female pups from the 30 mg/kg morphine treated group died at a higher rate than their male counterparts. The greater risk posed in the female pups to morphine exposure could be due to sexually dimorphic differences in placental development. A previous study found that the placenta of female spiny mice undergo a greater degree of vascularization compared to males throughout gestation (O'Connell, Moritz, Walker, & Dickinson, 2013). This difference may lead to a greater degree of drug exposure in female offspring compared to males. Additionally, these sex differences may be the result of an opioid-induced endocrinopathy in both the pregnant dams, as well as the pups (Seyfried & Hester, 2012). In future studies, we aim to record maternal behaviors during and after morphine treatment to improve our understanding of the role of sex and stress hormones on maternal care. Additionally, we plan to investigate the role the placenta may have on potential mechanisms related to withdrawal severity and survival in both male and female pups.

Prenatal opioid exposure also led to a significant decrease in body weight in male pups , this finding is similar to previous studies (Byrnes & Vassoler, 2017). While changes in body temperature have not been well characterized in rodent models of NOWS, a 2019 study showed that body temperature decreased following prenatal opioid exposure (Wallin et al., 2019). Additionally, studies in adult rodent models of opioid withdrawal have also reported a decrease

in body temperature (Belknap, 1989; Lipták et al., 2012). In contrast to these previous reports, the body temperature of spiny mice pups from the morphine exposed groups was significantly higher in both sexes. This difference between body temperatures compared to previous reports may be due to variations in the hormonal profiles between other rodent models and spiny mice. For example, prenatal opioid exposure has been shown to impact both the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system by altering the expression of endogenous opioids which are known to modulate a stress response (Byrnes & Vassoler, 2017; Drolet et al., 2001). Unlike other rodent species, the fetal adrenal gland and brain of spiny mice have been found to produce dehydroepiandrosterone (DHEA) (Lamers et al., 1986). Although the presence of DHEA has been shown to decrease body temperature when administered to rodents, chronic morphine has been shown to decrease the levels of dehydroepiandrosterone sulfate (DHEAS), a precursor to DHEA, in both males and females (Catalina, Milewich, Frawley, Kumar, & Bennett, 2002; Daniell, 2006). Also, previous studies in spiny mice have shown that the glucocorticoid cortisol is present when in other rodents, corticosterone is typically found (Lamers et al., 1986; Quinn et al., 2013). These differences in hormonal profiles could account for differences in the dysregulation of the HPA axis among rodent models and may give rise to the increase in body temperature of spiny mice which interestingly, closely resembles fever-like clinical symptoms observed in human infants experiencing NOWS (Kocherlakota, 2014).

Unlike most rodent species, spiny mice are precocial and are capable of walking shortly after birth (Brunjes, 1990). This unique characteristic allowed us to observe and measure withdrawal behaviors that are typically examined in adolescent and adult mice (Jones & Barr, 1995). For example, we measured wet dog shakes and jumping from PND 0 – 7. We observed an

increase in these withdrawal behaviors in pups from the dams treated with morphine. In male pups, wet dog shakes were greater in the 30 mg/kg morphine exposed group, whereas in females wet dog shakes were greater in 10 mg/kg morphine exposed group. Additionally, we observed an increase in jumping behavior in morphine exposed spiny mice. Interestingly, the number of jumps was higher in both male and females exposed to 10mg/kg morphine and this could be due to the effects on locomotion (motor skills). Previous studies have shown that morphine can have a biphasic effect on locomotion depending on the dose (Patti et al., 2005). Morphine can elicit a initial depression phase followed by hyperlocomotion phase, however, since our data is from spiny mice pups (0 – 7 days-old) the increase in jumping may also be related to the development of motor skills during the first seven days of life. Previous studies have demonstrated that morphine at various doses can elicit a hyperlocomotive effect, our data also show a biphasic effect of morphine on locomotor behavior in spiny mice with stimulant effects at 10 mg/kg and depressive effects at 30 mg/kg (Belknap et al., 1998). The exact mechanism for these morphine-mediated effects on motor skills are currently unknown. We also found that spontaneous opioid withdrawal in spiny mice resulted in symptoms such as wall climbing, face cleaning, tremors, and USV's which have previously been characterized in infant rodents (Barr & Wang, 1992; Ceger & Kuhn, 2000; K. L. Jones & Barr, 1995; H. Zhu & Barr, 2004).

In our study, we found sex differences in almost all of these behaviors with the most striking differences observed in tremor behavior and USV's. Tremors are often observed in human infants with NOWS which results from the dysregulation of the autonomic nervous system and the transmission of norepinephrine (Kocherlakota, 2014). Here, we discovered that pups from the morphine exposed groups experienced a significantly greater number of tremors. More specifically, male pups exposed to 30 mg/kg morphine experienced a greater number of

tremors. In contrast, females exposed to 10 mg/kg morphine experienced a greater number of tremors. Additionally, we found a marked sex difference between the number of USV's measured. During isolation or in times of distress, pups commonly emit USV's to elicit a dam retrieval response (Ehret, 2005; Elwood, 1979; Smotherman, Bell, Starzec, Elias, & Zachman, 1974). Previous studies have shown that opioids can reduce USV's (Carden, Barr, & Hofer, 1991) and when precipitated, opioid withdrawal can result in a greater number of USV's and is thought to be a measure of distress and/or dysphoria (Bell, Nitschke, Gorry, & Zachman, 1971; Covington & Miczek, 2003; Hofer & Shair, 1987). This increase in the number of USV's is now considered a marker of opioid withdrawal in preclinical models (Barr et al., 2011). Here, we report that male and female spiny mice pups from the morphine exposed groups emitted significantly fewer USV's between PND 0 - 7. Evidence of sex differences in USV's following opioid exposure was recently reported; male rodents emitted a greater number of USV's compared to female rodents (Robinson et al., 2019). Differences observed between male and female spiny mice pups may be attributed to sexually dimorphic transmission of norepinephrine from the locus coeruleus as the dysregulation of norepinephrine has been implicated in opioid addiction and withdrawal as well as in clinical symptoms of NOWS (Aston-Jones & Kalivas, 2008; Kocherlakota, 2014). Following prenatal morphine exposure, an increase in hypothalamic levels of norepinephrine and rate of turnover was observed in male rats. Whereas, females were found to have decreased levels of hypothalamic norepinephrine and turnover rate (Vathy et al., 1994). Additionally, previous studies have found that in untreated mice, males have been shown to emit a greater number of USV's compared to females (Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013). Thus, our results may be explained by inherent sex differences in USV's (Barr & Wang, 1992; Ceger & Kuhn, 2000; Jones & Barr, 1995; Zhu & Barr, 2004). Studies in

rodents have also shown that the emission of USV's changes during early postnatal development with the rate of USV's peaking around PND 7 and subsiding at approximately PND 14 (Elwood & Keeling, 1982). It is important to note that when comparing our findings to other studies, we should carefully consider the use of opioid antagonists used to precipitate opioid withdrawal and/or the postnatal drug regime used to mimic a third-trimester drug exposure. When taken together, it can be difficult to make direct comparisons of our results to those that also measured USV's at later postnatal time-points. Another key difference between other rodent models and spiny mice is that their ears and auditory structures are open and functional at birth which may have an impact on USV's (Brunjes, 1990; Ehret, 1983). Lastly, this is the first time that USV's have been studied in spiny mice, and we hope to gain more insight into this behavior as we learn more about this novel species.

We recognize that our preclinical model of NOWS has limitations. For example, one major limitation of our study was the treatment regime. Our study initiated drug treatment mid-gestation on GD 19. Commonly, women are already engaged in drug abuse before conception and continue throughout gestation and the postpartum periods. However, due to the challenges of confirming pregnancy in spiny mice without an ultrasound machine, we chose to begin treatment mid-gestation following the confirmation of pregnancy measured as a percentage of weight gain from baseline weights; a sustained 1% increase in body weight was indicative of successful pregnancy. Despite this limitation, we believe our study captures a very critical period of gestation that coincides with the peak time of brain development that takes place during the 3rd trimester of pregnancy (Semple et al., 2013). Human studies have shown that infants exposed to opioids during the 3rd trimester have a greater risk for developing NOWS compared to fetus exposed at an earlier period of pregnancy (Desai et al., 2015).

Additionally, the relatively small litter size and longer gestational period of spiny mice limited our ability to scale this study. Cages were checked daily at 08:00 hr. for new pups and without a 24 hr. video monitoring system we were unable to record the precise time of births. This may account for any inter- and intra-treatment groups variations in withdrawal behavioral outcomes measured during PND 0 - 7. Another limitation of our study is the dosing frequency. We injected once-daily to minimize the stress associated with handling and injections in pregnant dams. However, we could have injected twice daily but this could have also introduced more stress that could have impacted the health and behaviors of both dam and pups. Many previously published reports show the successful use of osmotic minipumps to deliver a constant dose of a drug. However, in our study due to the long gestation periods of spiny mice and the 10 and 30 mg/kg morphine doses used prevented us from utilizing mini-pumps. Additionally, mini pumps would not allow us to adjust the dose of morphine as the body weight of pregnant dams increased.

CONCLUSION

Taken together, our findings validate the use of spiny mice to investigate the effects of prenatal morphine exposure and introduces a novel preclinical model of NOWS. Prenatal morphine exposure affected the development of opioid withdrawal, and perhaps female pups showed more effects than male pups. Our results are consistent with previous research whereby chronic morphine exposure increased jumps, wet dog shakes, wall climbs, face cleaning, and tremors, all well-characterized behaviors associated with opioid withdrawal. Inconsistences in the literature may be due to the variety of doses used, different rodent species, and different treatment regimes. Future studies aim to investigate the long-term effects of prenatal opioid

exposure on learning and memory in spiny mice and determine cell-specific and molecular changes in the brain induced by prenatal opioid exposure during withdrawal. We are hopeful that our novel mouse model of NOWS will not only provide new insights into the unknown effects of prenatal drug exposure but also highlight the importance of using a more mature and developed rodent species like spiny mice in numerous areas of biomedical research.

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CHAPTER 5

LONG TERM EFFECTS OF PRENATAL MORPHINE EXPOSURE ON LEARNING AND MEMORY

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ABSTRACT

As opioid use among pregnant woman increases, the number of infants born with neonatal opioid withdrawal syndrome (NOWS) continues to rise. Although the short-term withdrawal symptoms are well characterized, the neuropathology behind NOWS and the long-term effects on behavior and memory are unclear. We previously reported withdrawal behaviors associated with prenatal opioid exposure in a novel rodent model using spiny mice. Using this unique rodent species, with an extended gestational period, we aimed to study the long-term effects of *in utero* morphine exposure on learning and memory. Dams were treated daily with saline or morphine 10 and 30 mg/kg S.C. beginning on G19 until day of birth, resulting in an exposure of 19-21 days. Y-maze assessments were conducted bi-weekly in offspring to assess spatial reference and working memory beginning at 1 month of age. A decrease in spontaneous alternation was observed at 1 month of age in both male and female offspring prenatally exposed to morphine. Decreased spontaneous alternation persisted in morphine exposed males until 3 months of age. Morphine exposed males made fewer visits to the blocked arm during the spatial recognition test at 1 month of age. However, females prenatally exposed to morphine made fewer visits to the blocked arm from 1.5-3.0 months of age. Our results show prenatal opioid exposure leads to long-term deficits in spatial reference and working memory that persist from adolescence into adulthood in spiny mice. Additionally, we observed sex differences in these behaviors. Further studies are in progress to determine underlying cellular changes within the brain that may account for these differences.

INTRODUCTION

As the opioid crisis continues to grow around the globe, the United States has seen a five-fold increase in infants suffering from neonatal abstinence syndrome (NAS) between 2004-2014, and reaching an incidence rate of 8.8 cases per 1000 births in 2016 (Leech, Cooper, McNeer, Scott, & Patrick, 2020; Winkelman et al., 2018). An estimated 26,000-32,000 infants were diagnosed with NAS in 2016 (Agency for Healthcare Research and Quality 2020; Doherty et al., 2020; Leech et al., 2020). Unfortunately, a recent study suggests that due to deficiencies in medical tracking these estimates do not reflect the true magnitude of the consequences associated with maternal substance abuse and the number of infants suffering from NAS in the US (Doherty et al., 2020). Neonatal abstinence syndrome is a postnatal drug withdrawal syndrome experienced by infants prenatally exposed to drugs. Most commonly, opioid exposure is associated with the presentation of NAS, and therefore is now more appropriately referred to as neonatal opioid withdrawal syndrome (NOWS). The clinical presentation of NOWS consists of a constellation of signs and symptoms including irritability, inconsolability, high pitched cry, tremors, gastrointestinal disturbances, poor feeding, fever, and in severe cases seizures (Kocherlakota, 2014). Although the withdrawal symptoms of NOWS are well characterized, the short- and long-term neurodevelopmental and behavioral consequences of prenatal opioid exposure are not understood. More specifically, the impact of prenatal opioid exposure on learning and memory is currently unknown.

Many of the studies conducted investigating the consequences of prenatal drug exposure focus on developmental deficits at birth, infancy, and early childhood. Due to limited ability to track children throughout growth, foster care, and poor healthcare follow-up it is difficult to understand the long term challenges these children will face. However, there are a few studies

that provide evidence that prenatal drug exposure does in fact lead to long term deficits. One study assessed prenatally exposed children at 1, 2, 3, 4.5, and 8.5 years of age. They found that prenatal opioid exposure lead to decreased cognitive abilities in both male and female children throughout childhood. However, their results indicated a significant gender bias. More specifically, males showed significantly lower IQ scores at all time points compared to the control group, whereas females only presented with significantly IQ scores at 8.5 years of age (Nygaard, Moe, Slinning, & Walhovd, 2015). In 2016, a study was published providing the first long-term study of school performance in children who were diagnosed with NOWS. They found that children with NOWS had poorer performance on standardized tests beginning in grade school which persisted to high school (Oei et al., 2017). These findings suggest that prenatal opioid exposure does have a lasting impact on neurodevelopment in children diagnosed with NOWS.

However, children born to mother's dependent on opioids are at an increased risk of experiencing environmental adversities such as poverty, poor nutrition, and decreased quality of perinatal care which may also increase the risk for long-term developmental deficits (Dawe, Harnett, Staiger, & Dadds, 2000; Wouldes & Woodward, 2010). Additionally, mothers that suffer from substance abuse disorders often possess poor parenting skills, compromised caregiving abilities, and are at higher risk for developing psychiatric disorders (Davie-Gray, Moor, Spencer, & Woodward, 2013; Hatzis et al., 2017). Some studies suggest that suboptimal caregiving environment does in fact affect development in children diagnosed with NOWS and improvement of the postnatal environment may help overcome vulnerabilities experienced by these children (Baar & Graaff, 1994; Messinger et al., 2004). However, other studies suggest that despite being adopted at a young age, children and infants diagnosed with NOWS still

experienced decreased mental abilities, cognitive and psychomotor deficits, and showed more signs of attention deficit disorders when compared to controls throughout infancy, early childhood, and adolescence (Baar & Graaff, 1994; Moe & Slinning, 2001; Ornoy et al., 2010; Slinning, 2004; Strauss, Starr, Ostrea, Chavez, & Stryker, 1976). These confounding variables make it difficult to determine the direct relationship of prenatal opioid exposure with impaired long-term neurodevelopment. As a result, continued animal studies are needed to bridge the gaps in our understanding of the consequences of NOWS, specifically regarding the long-term developmental outcomes and its effect on learning and memory.

As previously stated, current rodent models used to study prenatal opioid exposure have limited clinical translatability due to short gestational periods, large litter sizes, and primary organogenesis occurring postnatally. In our previous study, we introduced a novel preclinical rodent model of NOWS validating the use of the rodent species *Acomys cahirinus*, more commonly known as spiny mice, to further our understanding of the consequences of prenatal opioid exposure on the developing fetus (Stevens & Mohan, 2021). Spiny mice possess several unique biological characteristics including lengthened gestational periods, small litter sizes, and increased in utero organogenesis (Brunjes, 1990; Dickinson & Walker, 2007). Additionally, previous investigators have highlighted the fact that spiny mice have a lengthy life expectancy with evidence that they can live for more than three years in captivity (Haughton et al., 2016). This makes them an ideal species not only to study the short-term consequences of prenatal opioid exposure, but also its the long-term consequences. Here we aim to use this novel rodent species to investigate the impact of prenatal opioid exposure, and subsequent opioid withdrawal, on long term neurodevelopment and its effect on learning and memory in spiny mice offspring.

METHODS

Breeding

All spiny mice used in this study were obtained from our in-house breeding colony maintained at Purdue University, Fort Wayne, IN. All experiments were conducted per the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Purdue Institutional Animal Care and Use Committee at Purdue University. Spiny mice were bred male: female (1:1) starting between 9 - 12 weeks of age. Spiny mice do not produce a vaginal plug after mating, therefore the date of birth for the first litter was used to determine gestational age (days) of experimental pups. Gestational age was determined from the time of post-partum conception (i.e. mating at 24 h after delivery of a previous litter), as previously described (Hayley Dickinson et al., 2005b).

Morphine Treatment

On gestational day (G) 19, dams were separated from their male partner and remained isolated throughout treatment. Dams were briefly anesthetized using 2% isoflurane and treated (S.Q.) once-daily (between 09:00 – 10:00 h) with either saline (N = 8) or morphine sulfate (Spectrum Chemical, M1167) at two different concentrations, 10 and 30 mg/kg (N = 9 / dose). Upon birth, all pups (total N's: saline = 24; 10 mg/kg morphine = 24; and 30 mg/kg morphine = 21) were evaluated for signs of opioid withdrawal from PND 0 – 7, results were previously reported (Stevens & Mohan, 2021). Pups remained with the dam until day of weaning at postnatal day (PND) 14 at which time they were ear tagged and placed into fresh cages. Pups were housed with littermates of the same sex. Pups were assigned to one of the following age groups PND 30 or PND 90, and aged for behavioral testing. The timeline of experiments is

shown in (Fig. 5.1). All mice were maintained on a 12h light / dark cycle (lights on at 06:00 h) in a temperature (24 - 26 °C) and humidity (40 - 70%) controlled environment. Food and water were available *ad libitum*.

Experimental Design

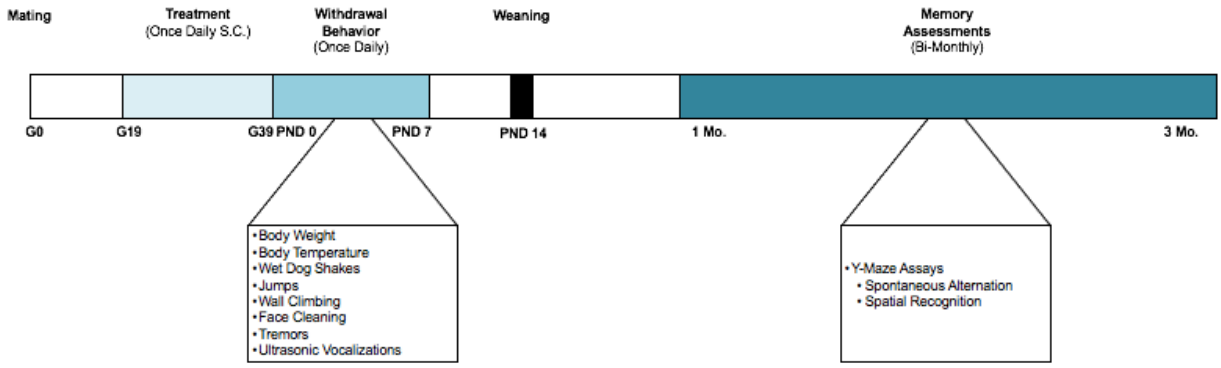

Fig. 5.1 Experimental Timeline
Pregnant dams were treated once daily with saline or morphine (10mg/kg or 30 mg/kg) S.C.. Behavioral testing was conducted daily from PND 0-7 on spiny mice pups to assess symptoms of withdrawal. Pups remained with dams until day of weaning at PND 14. Beginning at one month of age, Y-maze assays were conducted bi-monthly until three months of age to assess changes in learning and memory.

Behavioral Procedures

All behavioral testing was conducted during between 0800 – 1200. Mice were placed within the testing room for a minimum of 30 minutes to acclimate each day before testing. The temperature within the testing room was maintained at a constant temperature of 24 °C and humidity (40-70%). Prior to testing, each mouse was weighed and body weight recorded. Spiny mice were recorded during testing to allow for more accurate post-testing observations (blinded) and data collection. The behavioral assays were performed in the order in which they are described below.

Spontaneous Alternation Test

Beginning at PND 30, adolescent mice were assessed for spatial working memory performance. Mice were placed in one arm of a y-maze (35cm arm length, 5cm arm width, 20cm arm height, Maze Engineers, Glenview IL) facing away from the center of the maze. Mice were allowed to freely explore each of the three arms of the y-maze for five minutes. The animal's exploration of the maze was video recorded. Once completed, mice were removed from the y-maze and placed back in their home cage. The y-maze was cleaned with 70% ethanol between each trial to ensure the removal of odor cues, which has been shown to influence alternation rate in rodents (Still & Macmillan, 1975). Video recordings were reviewed by trained experimenters that were blinded to treatment. The order and number of arm choices were manually recorded. An entry was indicated when the mouse placed all four limbs inside an arm and was facing forward. Entries made by backing into the arm were not scored. Three consecutive choices of three different arms were counted as a spontaneous alternation. % spontaneous alternation was calculated as the number of spontaneous alternations divided by the total number of arm entries minus 2 multiplied x 100 as shown below.

$$\frac{\#\ spontaneous\ alternations}{Total\ number\ of\ arm\ entreis - 2} \times 100$$

For example, if the arm entry order was: A B C A B B C A B A C B, the experimenter would score a total of 7 spontaneous alternations (ABC, BCA, CAB, BCA, CAB, BAC, ACB). With a total of 12 arm entries, the % alternation for this trial would be 70%. Spontaneous alternation behavior was assessed bi-monthly up to 3 months of age.

Spatial Recognition Test

Seven days after spontaneous alternation testing, spiny mice were assessed for spatial reference memory, using a spatial recognition test. This test was conducted in two phases. During the first phase, one arm of the y-maze was blocked while the other two remained open. Mice were placed in one of the open arms facing away from the center of the maze. Mice were allowed to freely explore the two open arms of the y-maze for 5 minutes. At the end of phase one, mice were removed from the maze and returned to their home cage for one hour. During this time, the y-maze was cleaned with 70% ethanol followed by water and dried to remove any olfactory cues. Next, the partition was removed from the maze so that all three arms of the y-maze were open. At the end of one hour, mice were placed back into the y-maze and allowed to freely explore all three arms of the maze for a total of 5 minutes. The animal's exploration of the maze during phase two was video recorded. After testing was complete, recordings were analyzed and arm choices were manually recorded. An entry was indicated when the mouse placed all four limbs inside an arm and was facing forward. Entries made by backing into the arm were not scored. % recognition was used as a measure of spatial recognition and was calculated as the number of visits made to the previously blocked arm divided by the total number of arm visits as shown below. Spatial recognition assessments were conducted bi-monthly up to 3 months of age.

$$\frac{\#\ Entries\ in\ blocked\ arm}{Total\ number\ of\ arm\ entreis} \times 100$$

Data Analysis

All data are presented as mean ± SEM. Spontaneous alternation, % entries to blocked arm, and total number of arm entries at one month of age were assessed using an ordinary one-way ANOVA with Tukey's post hoc test, and treatment as the factor. Spontaneous alternation, % entries to blocked arm, and total number of arm entries in adult mice (1.5- 3.0 months) were assessed using a two-way repeated-measures ANOVA with Tukey's post hoc test, with treatment and age as factors. Body weight was assessed using two-way repeated-measures ANOVA with Tukey's post hoc test, with treatment and age as factors. Sex differences within treatment groups for each test were analyzed using two-way repeated-measures ANOVA with Sidak's post hoc test with sex and age as factors. All data were analyzed with GraphPad Prism© software version 8.0 (San Diego, CA, USA) and differences were considered significant for $P < 0.05$.

RESULTS

Spontaneous Alternation Test

Prenatal morphine exposure led to a decrease in spontaneous alternation among adolescent offspring. At one month of age males from both morphine (10 and 30 mg/kg) exposure groups had a significantly lower % alternation compared to saline (55.29 ± 2.51 and 60.03 ± 2.34 vs. saline: 70.01 ± 2.98) ($P < 0.05$) (Fig. 5.2a). There were no significant differences in the total number of entries among all exposure groups (Fig. 5.2b).

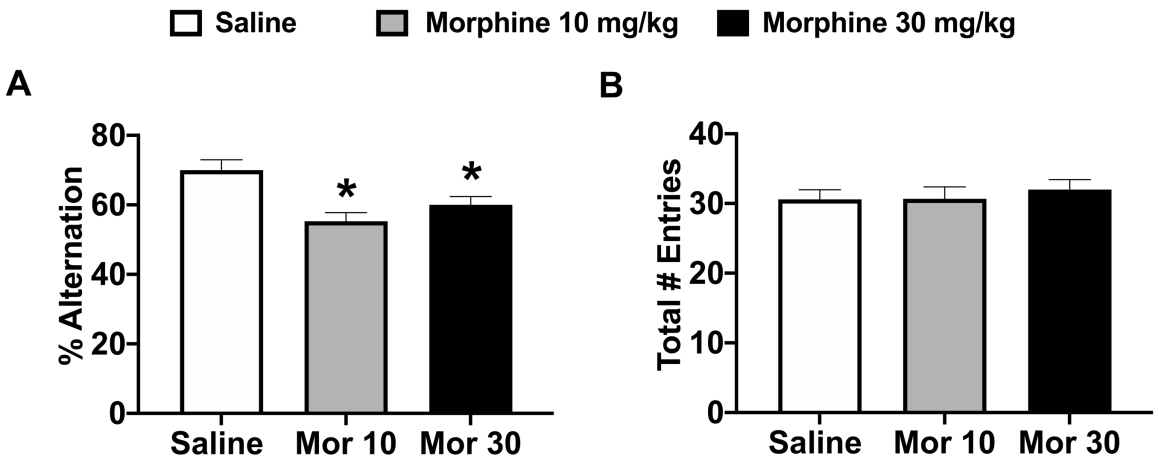

Fig. 5.2 Male Spontaneous Alternation at 1 Month of Age
(a) At 1 month of age, males exposed to morphine (10 mg/kg: N= 12 and 30 mg/kg: N= 12) had a significantly lower % alternation compared to saline-exposed controls (N= 12). (b) There was no significant difference in total number of arm entries. Data are represented as mean totals (± SEM). An asterisk symbol (* vs. saline) is used to denote significance between morphine exposure groups and saline, $P < 0.5$.

Similarly, female mice exposed to 10 mg/kg of morphine had a significantly lower % alternation compared to saline and morphine 30 mg/kg mice at one month of age (53.05 ± 1.87 vs. saline: 65.79 ± 1.87 morphine 30 mg/kg: 62.16 ± 3.12) ($P < 0.05$) (Fig. 5.3a). There were no significant differences in the total number of entries among exposure groups (Fig. 5.3b). There were no sex differences observed in % alternation or total number of arm entries.

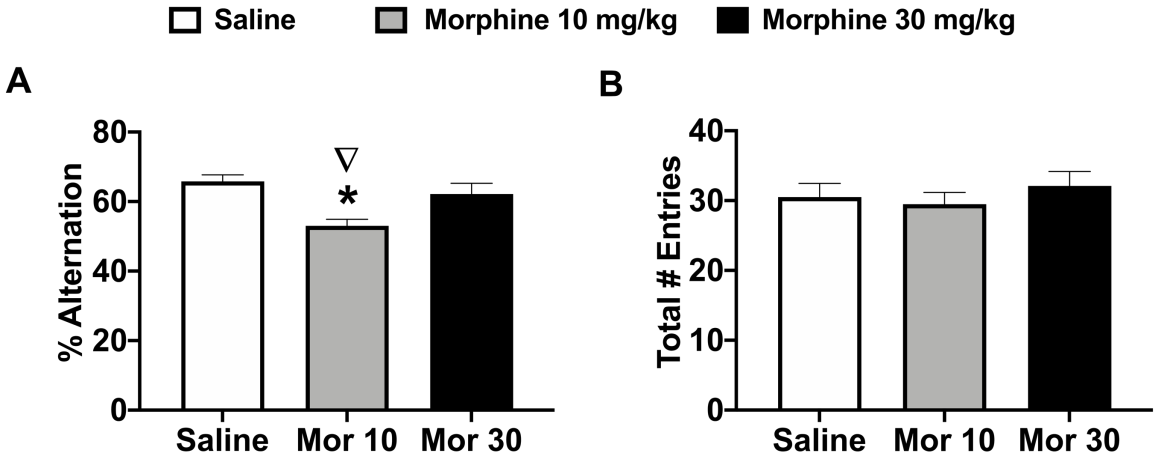

Fig. 5.3 Female Spontaneous Alternation at 1 Month of Age
(a) At 1 month of age, females exposed to 10 mg/kg morphine (N= 12) had a significantly lower % alternation compared to saline-exposed controls (N= 12) and 30 mg/kg morphine exposed mice (N=12). (b) There was no significant difference in total number of arm entries between exposure groups. Data are represented as mean totals (± SEM). An asterisk symbol (* vs. saline) or nabla symbol (∇ vs. morphine 30 mg/kg) is used to denote significance between exposure groups, $P < 0.5$.

Adult male mice exposed to morphine (10 mg/kg and 30 mg/kg) showed a decrease in % alternation compared to saline at 1.5-3.0 months of age. However, these differences were not found to be significant ($p > 0.5$) (Fig. 5.4a). Males exposed to morphine (10 mg/kg and 30 mg/kg) made a greater number of total arm entries compared saline at 1.5-2.5 months of age (Fig. 5.4b).

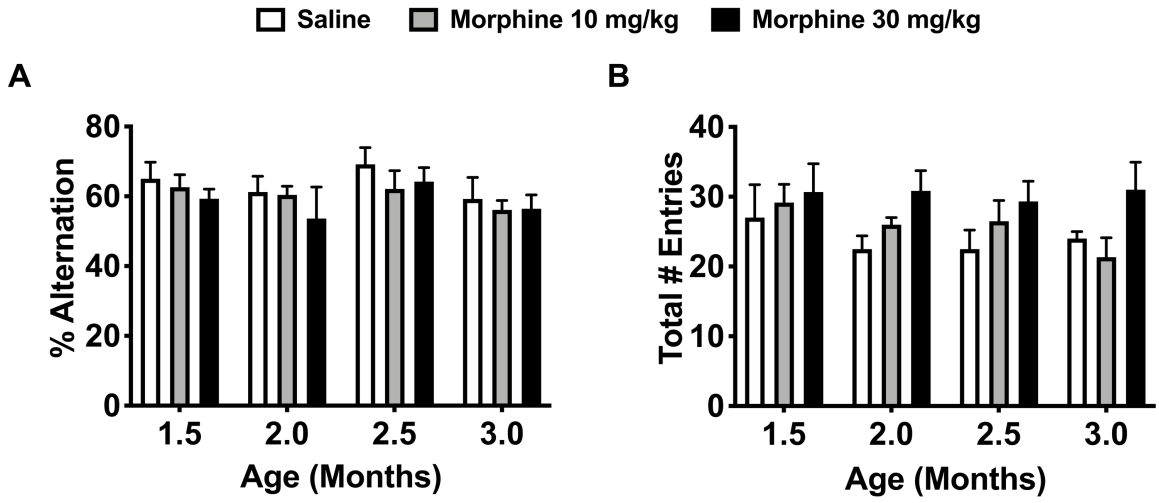

Fig. 5.4 Male Spontaneous Alternation at 3 Months of Age
(a) At 3 months of age, there were no significant differences in % alternation in male spiny mice exposed to morphine (10 mg/kg: N= 6, 30 mg/kg: N=6) and saline-exposed controls (N= 6) (b) There was no significant difference in total number of arm entries between exposure groups. Data are represented as mean totals (± SEM).

There were no significant differences in % alternation or number of arm entries in female mice at 1.5-3.0 months of age (Fig. 5.5a-b). Additionally, there were no sex differences observed in % alternation or total number of arm entries.

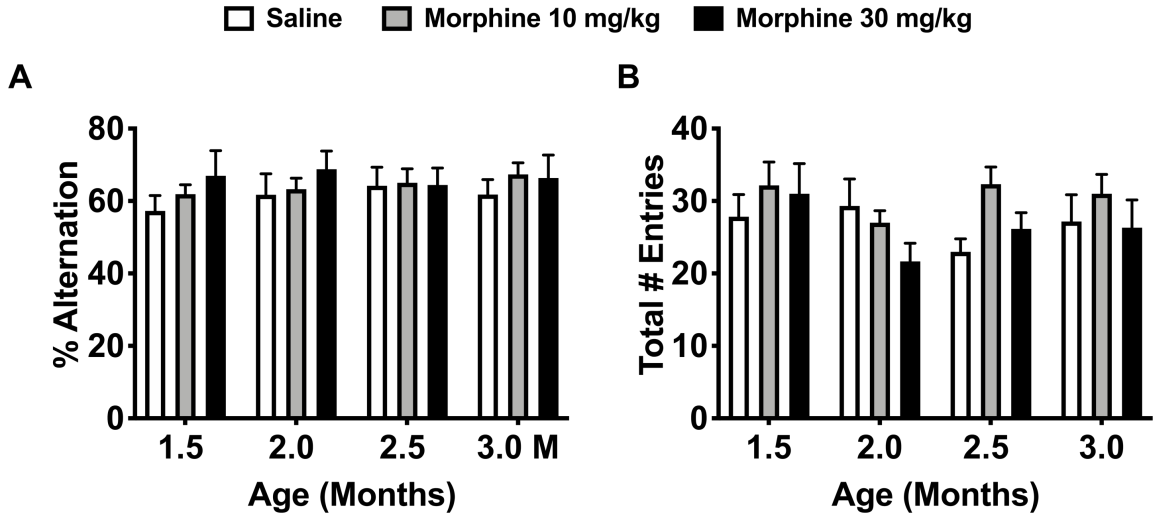

Fig. 5.5 Female Spontaneous Alternation at 3 Months of Age
(a) At 3 months of age, there were no significant differences in % alternation in female spiny mice exposed to morphine (10 mg/kg: N= 6, 30 mg/kg: N=6) and saline-exposed controls (N= 6) (b) There was no significant difference in total number of arm entries between exposure groups. Data are represented as mean totals (± SEM).

Spatial Recognition Test

Prenatal morphine exposure led to a decrease in the number of entries made in the blocked arm in adolescent male spiny mice. Although significance was not observed ($P > 0.5$), males exposed to morphine (10 mg/kg and 30 mg/kg) on average made fewer visits to the previously blocked arm compared to saline (Fig. 5.6a). In addition, males exposed to 10 mg/kg morphine made significantly fewer number of total arm entries compared to 30 mg/kg morphine exposed mice (20.33 ± 1.47 vs. 28.58 ± 1.64) ($P < 0.05$) (Fig. 5.6b).

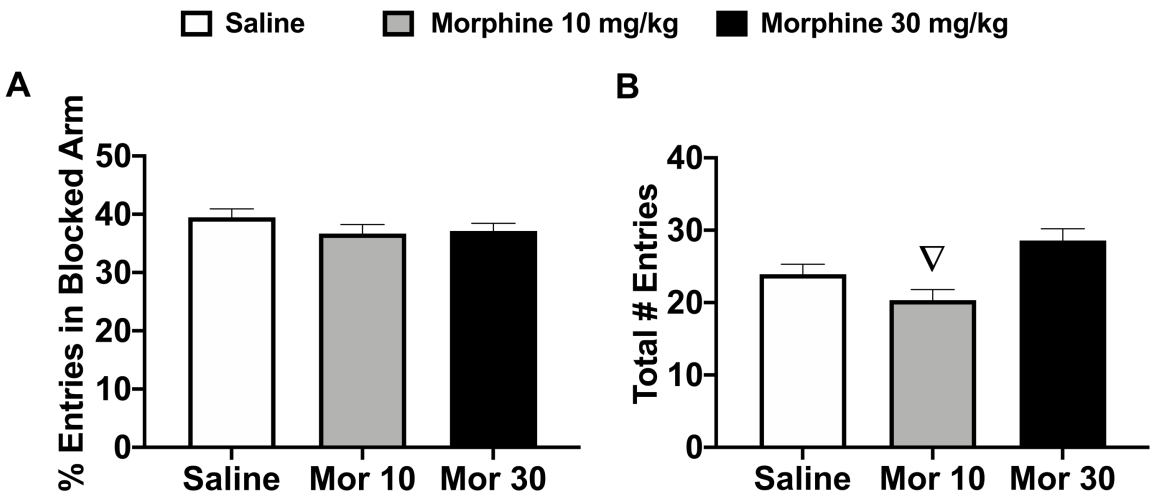

Fig. 5.6 Male Spatial Recognition at 1 Month of Age
(a) At 1 month of age, there were no significant differences in the percentage of entries in the blocked arm in males exposed to morphine (10 mg/kg: N= 12, 30 mg/kg: N=12) and saline-exposed controls (N= 12). (b) Males exposed to morphine10 mg/kg had a significantly less total number of arm entries compared to 30 mg/kg morphine exposed mice. Data are represented as mean totals (± SEM). A nabla symbol (∇ vs. morphine 30 mg/kg) is used to denote significance between morphine treatment groups, $P < 0.5$.

There were no significant differences in the number of entries to the blocked arm or total number of arm entries in adolescent female spiny mice (Fig. 5.7a). However, females exposed to morphine (10 mg/kg and 30 mg/kg) made fewer number of total arm entries compared to saline ($P > 0.5$) (Fig. 5.7b). Additionally, there were no sex differences observed.

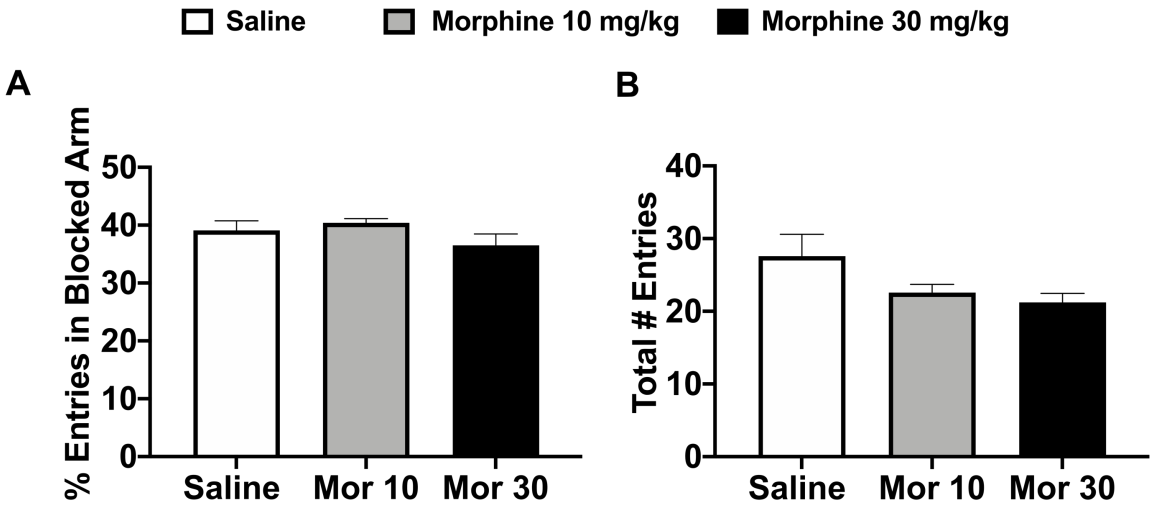

Fig. 5.7 Female Spatial Recognition at 1 Month of Age
(a) At 1 month of age, there were no significant differences in the percentage of entries in the blocked arm in females exposed to morphine (10 mg/kg: N= 12, 30 mg/kg: N=12) and saline-exposed controls (N= 12) or (b) the total number of arm entries. Data are represented as mean totals (± SEM).

Prenatal morphine exposure led to a decrease in the number of entries to the previously blocked arm in adult mice. There were no significant differences in the number of entries to the blocked arms in adult males (Fig. 5.8a). However, males exposed to 30 mg/kg morphine made a greater number of total arm entries compared to males exposed to saline and morphine 10 mg/kg ($P > 0.5$) (Fig. 5.8b).

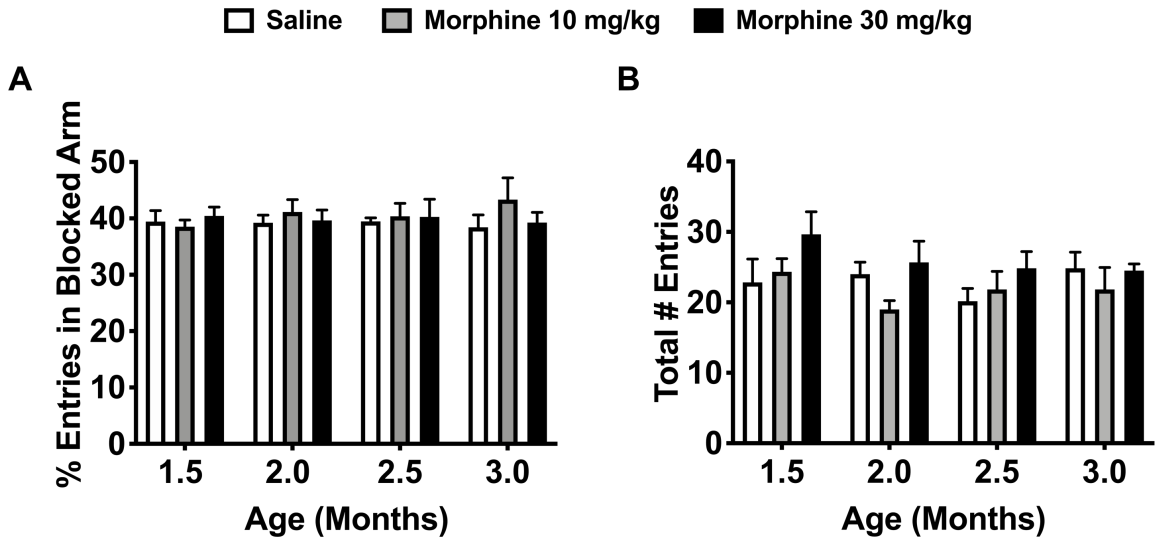

Fig. 5.8 Male Spatial Recognition at 3 Months of Age
(a) At 3 months of age, there were no significant differences in the percentage of entries in the blocked arm in adult male spiny mice exposed to morphine (10 mg/kg: N= 6, 30 mg/kg: N=6) and saline-exposed controls (N= 6) or (b) total number of arm entries. Data are represented as mean totals (± SEM).

In contrast, adult females exposed to morphine (10 mg/kg and 30 mg/kg) made fewer visits to the previously blocked arm compared to saline at 1.5-3.0 months. At 2.5 months of age, females exposed to 10 mg/kg morphine made significantly fewer visits to the previously blocked arm compared to saline and morphine 30 mg/kg treated mice (37.90 ± 1.32 vs. saline: 48.13 ± 2.52 morphine 30 mg/kg: 42.31 ± 2.20) ($P < 0.05$) (Fig. 5.9a). Additionally, at 2.5 months of age, females exposed to 10 mg/kg morphine made a significantly greater number of total arm entries compared to saline and morphine 30 mg/kg treated mice (28.17 ± 2.36 vs. saline: 19.67 ± 1.91 morphine 30 mg/kg: 18.50 ± 1.31) ($P < 0.05$) (Fig. 5.9b). There were no sex differences observed.

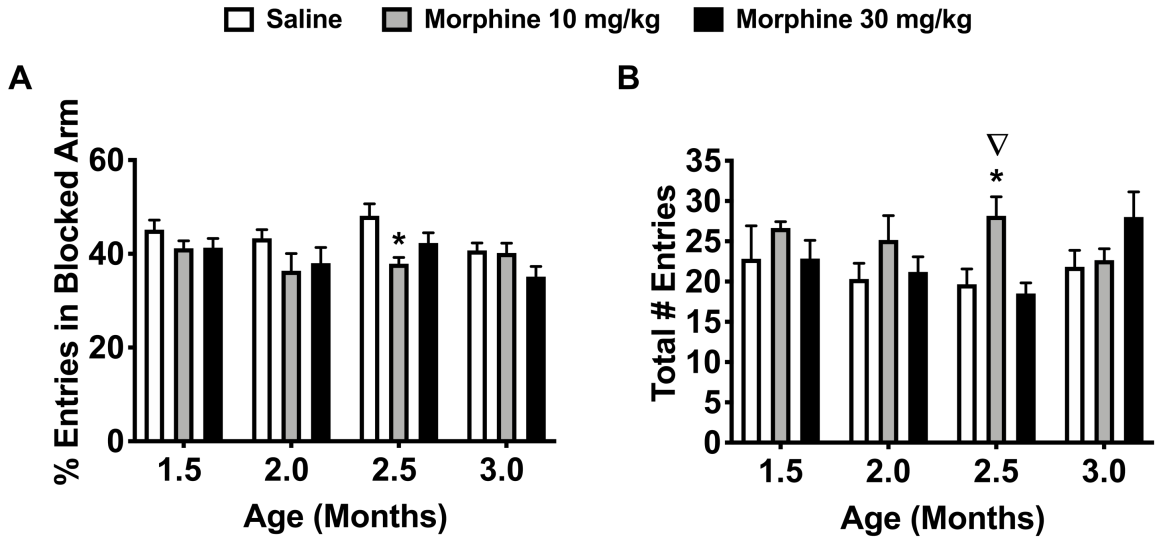

Fig. 5.9 Female Spatial Recognition at 3 Months of Age
(a)At 3 months of age, adult female spiny mice exposed to 10 mg/kg morphine (N= 6) made significantly fewer visits to the blocked arm compared to saline-exposed controls (N= 6). (b) Females exposed to 10 mg/kg morphine (N=6) had significantly greater total number of arm entries compared to 30 mg/kg morphine (N=6) treated mice and saline-exposed controls (N=6). Data are represented as mean totals (± SEM). An asterisk symbol (* vs. saline) or nabla symbol (∇ vs. morphine 30 mg/kg) is used to denote significance between treatment groups, $P < 0.5$.

Body Weight

Prenatal morphine exposure did not affect body weight in adolescent offspring at one month of age in male (Fig. 5.10) or female (Fig. 5.11) spiny mice.

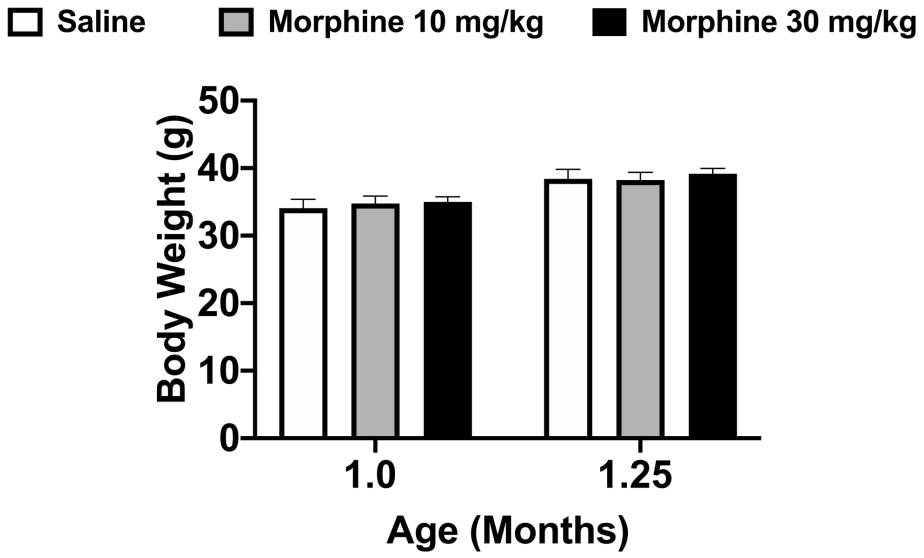

Fig. 5.10 Male Body Weight at 1 Month of Age
At 1 month of age, there was no significant difference in body weight (g) among morphine exposed mice (10 mg/kg: N=12, 30 mg/kg: N=12) and saline-exposed controls (N=12) in male spiny mice. Data are represented as mean totals (± SEM).

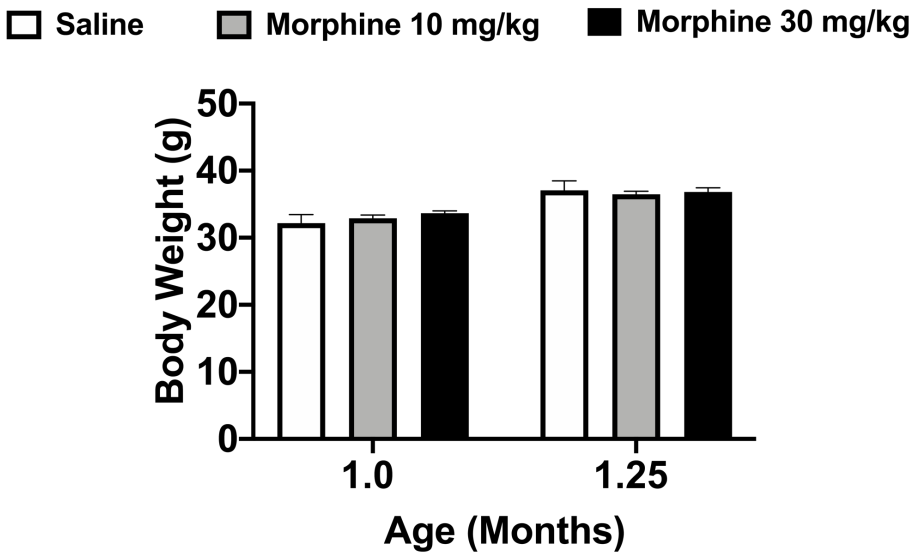

Fig. 5.11 Female Body Weight at 1 Month of Age
At 1 month of age, there was no significant difference in body weight (g) among morphine exposed mice (10 mg/kg: N=12, 30 mg/kg: N=12) and saline-exposed controls (N=12) in female spiny mice. Data are represented as mean totals (± SEM).

However, prenatal morphine exposure led to a decrease in body weight among adult offspring. Males exposed to morphine (10 mg/kg and 30 mg/kg) had lower body weights compared to saline mice at 1.5-3.0 months of age ($P > 0.5$) (Fig. 5.12).

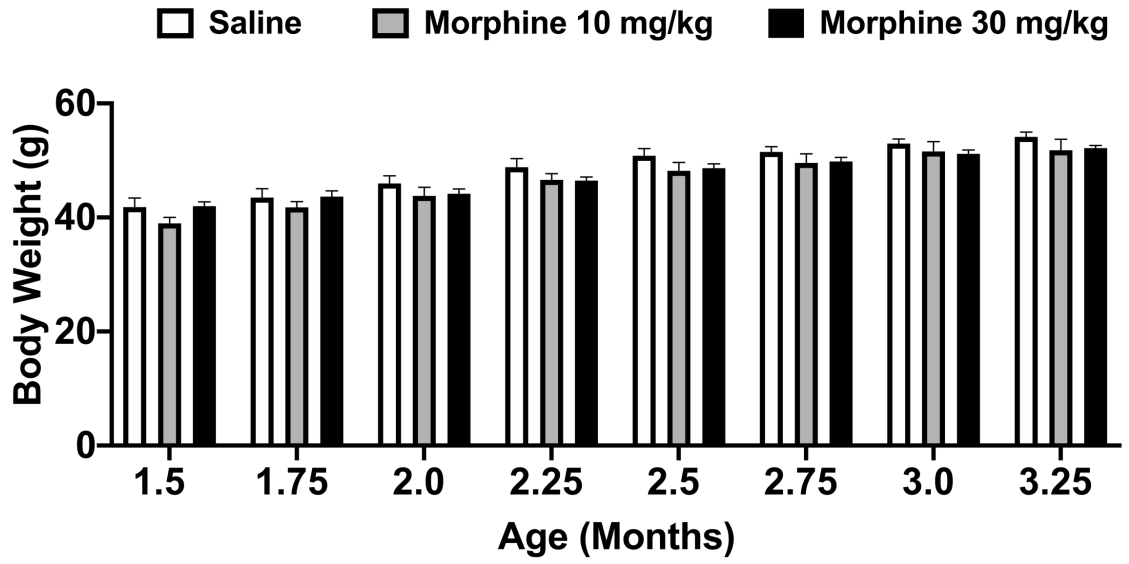

Fig. 5.12 Male Body Weight at 3 Months of Age
At 3 months of age, there was no significant difference in body weight among morphine exposed mice (10 mg/kg: N=12, 30 mg/kg: N=12) and saline-exposed controls (N=12) in female spiny mice. Data are represented as mean totals (± SEM).

Additionally, females exposed to morphine (10 mg/kg and 30 mg/kg) had lower body weights compared to saline exposed mice. More specifically, females exposed to 30 mg/kg morphine had significantly lower body weights compared to saline exposed mice at 2.25, 2.5, 2.75, and 3.0 months of age (42.33 ± 1.86, 44.33 ± 2.40, 45.00 ± 2.08, 46.00 ± 2.00 vs. saline: 50.67 ± 2.38, 52.33 ± 2.54, 52.50 ± 2.01, 52.50 ± 1.84) ($P > 0.5$) (Fig. 5.13).

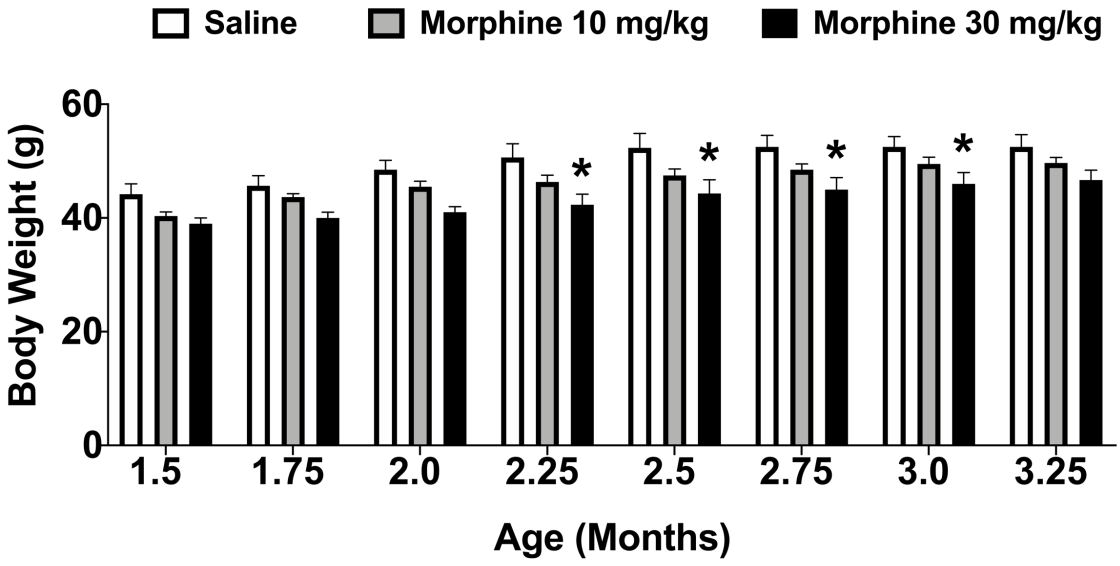

Fig. 5.13 Female Body Weight at 3 Months of Age

At 2.25, 2.5, 2.75, and 3.0 months of age morphine exposed mice (10 mg/kg: N=12, 30 mg/kg: N=12) had significantly lower body weights (g) compared to saline-exposed controls (N=12) in female spiny mice. Data are represented as mean totals (± SEM). An asterisk symbol (* vs. saline) is used to denote significance between treatment groups, $P < 0.5$.

DISCUSSION

The long-term impact of prenatal opioid exposure and opioid withdrawal on behavior and neurocognition largely remain unknown. Using spiny mice, we studied the long-term effect of prenatal morphine exposure, and subsequent withdrawal, on adolescent and adult offspring. By testing these mice at regular intervals throughout development we hope to gain understanding of the impact of prenatal opioid exposure and development of NOWS on learning and memory. Our study showed that prenatal opioid exposure and opioid withdrawal has a lasting negative impact on learning and memory, evidenced by decreased y-maze performance in both male and female spiny mice that persisted into adolescence and adulthood.

The Y-maze can be used to assess short term memory in rodents (Kraeuter, Guest, & Sarnyai, 2018). Here, we utilized two Y-maze assessments to measure the impact of prenatal morphine exposure and neonatal withdrawal on spatial working memory and reference memory in adolescent and adult spiny mice. Y-maze assessments rely on the natural curiosity and exploratory behavior of rodents (Lalonde, 2002). Many of the previous studies that assessed memory following prenatal opioid exposure utilized behavioral assays such as the Morris water maze, radial arm maze, and T-maze (Davis et al., 2010; Schrott, Franklin, & Serrano, 2008). Although these assays are well established as methods to measure memory, they rely on food restriction, arm baiting, and increased stress to elicit performance in the maze. Here we were able to assess memory without the use of food restriction or arm baiting to motivate spiny mice offspring.

Beginning at adolescence, at one month of age, spatial working memory was assessed by measuring spontaneous alternation in a Y-maze. During spontaneous alternation testing, mice are allowed to freely explore all three arms of the Y-maze. As they navigate through the maze, the

animal must remember the arm it has previously visited. A mouse with good spatial working memory, will typically enter arms that were less recently visited (Kraeuter et al., 2018). We observed that prenatal morphine exposure, and subsequent neonatal withdrawal, had a negative impact on spatial working memory in both male and female spiny mice. Males exposed to morphine exhibited a significantly lower number of Y-maze alternations compared to saline treated males. However, in females, this decrease in Y-maze alternations was only observed in the 10 mg/kg morphine exposed group, compared to saline and morphine (30 mg/kg) exposed females. In addition, there were no differences in the total number of arm entries between exposure groups in males or females. These findings were similar to other studies which reported a decrease in spatial working memory performance following prenatal opioid exposure (Davis et al., 2010; Lin et al., 2009; Schrott et al., 2008; Steingart et al., 2000; Yanai et al., 2000) . Additionally, these results are similar to those seen in adult studies of morphine exposure which resulted in decreased spontaneous alternation (Kitanaka et al., 2015).

Assessment of spatial working memory were conducted bi-monthly until three months of age. We found a trend of decreased spontaneous alternation behavior in morphine exposed males that continued from 1.5 months to 3 months of age (not statistically significant). These differences suggest that deficits in spatial working memory in male spiny mice persisted from adolescence (1 month of age) to adulthood (3 months of age). Additionally, during this time period, a trend of increased number of arm entries were observed in morphine exposed male mice compared to saline. This could be indicative of observed hyperactivity within these mice. In contrast, this deficit in spatial working memory from 1.5 - 3 months of age was not observed in females exposed to morphine.

Spatial reference memory was also assessed beginning at one month of age using a second two-trial Y-maze test. Spiny mice underwent this second Y-maze test seven days after the spontaneous alternation test. If reference memory was intact, mice would recognize the arm that it was not able to explore during the first trial, and visit this novel arm more frequently than the other two arms of the Y-maze (Kraeuter et al., 2018). We found that prenatal morphine exposure and neonatal withdrawal did not result in a decrease in spatial reference memory in adolescent male or female spiny mice at one month of age. Our results oppose previous findings that suggest prenatal opioid exposure led to decreased spatial reference memory in adolescent rats (Niu et al., 2009). It is likely that the inter-trail interval (ITI) of one hour between testing sessions was not long enough to observe memory deficits in adolescent spiny mice. Future studies may require increasing the ITI to determine if there are any deficits in spatial reference memory at one month of age. Additionally, we did not observe any differences in adult male spiny mice from 1.5 - 3 months of age.

However, in adult female spiny mice exposed to morphine we observed a decrease in spatial reference memory, indicated by fewer visits to the blocked arm. More specifically, at 2.5 months of age females exposed to 10 mg/kg morphine made significantly fewer visits to the previously blocked arm compared to saline treated mice. In addition, females exposed to 10 mg/kg morphine made a significantly greater total number of arm entries compared to both saline and 30 mg/kg morphine exposed mice. This indicates that despite more arm entries, these mice did not choose to explore the novel arm. These findings are similar to previous studies that also found a decrease in spatial reference memory in adult mice when assessed using the radial arm maze (Davis et al., 2010; Schrott et al., 2008). Although we obtained similar results with a deficit in spatial reference memory, the radial arm maze requires food restriction, baiting of maze arms

and food rewards. Here we utilized the Y-maze, which does not involve any food-based reward or motivation making this a more preferable assessment that measures spatial reference memory without additional variables being introduced to the testing scenario.

Successful navigation of the Y maze and generation of spontaneous alternation have been linked to several areas of the brain including the hippocampus, prefrontal cortex, and basal ganglia (Lalonde, 2002; Retailleau, Etienne, Guthrie, & Boraud, 2012; Sarnyai et al., 2000; Swonger & Rech, 1972). In this study, pregnant dams were treated daily with morphine (10 mg/kg or 30 mg/kg) between G 19 - G 40 leading to mid and late term gestational opioid exposure. Late gestation is a critical time period for hippocampus and cortical development (Bayer, Altman, Russo, & Zhang, 1993). Endogenous opioids are known to have a marked effect on the activity of neurons within the hippocampus including modulation of neurogenesis, as well as playing a role in learning and memory (Drake et al., 2007; Sargeant et al., 2008; Simmons & Chavkin, 1996; Y. Zhang et al., 2016). Additionally, previous animal studies have shown that prenatal opioid exposure has several effects on hippocampal development including changes in activity and decreased expression of NMDA receptors, altered levels of nerve growth factors such as BDNF, decreased expression of PSD-95, increased expression of apoptotic mediators, and decreased long term potentiation (Lin et al., 2009; Nasiraei-Moghadam et al., 2013; Niu et al., 2009; Schrott et al., 2008; Villarreal et al., 2008; Yang et al., 2000). Many of these effects were correlated with deficits in spatial working and reference memory. Collectively, these findings provide evidence that prenatal opioid exposure leads to long term changes in neural pathways critical for hippocampal based learning (Byrnes & Vassoler, 2017).

Previously, we found that prenatal opioid exposure led to a significant reduction in body weight of male offspring but not females during the early postnatal period (Stevens & Mohan,

2021). To assess any long-term trends in weight gain following prenatal opioid exposure body weight was measured prior to Y-maze testing each week. There were no significant differences in weight gain among treatment groups in male or female mice at one month of age. However, a trend towards a decrease in body weight was observed in morphine exposed mice that persisted throughout adulthood in both male and female spiny mice. More specifically, females exposed to 30 mg/kg morphine had a significantly lower body weight from 2.25 - 3.25 months of age. These findings are similar to a previous study which also reported a decrease in body weight in juvenile and adult offspring following prenatal morphine exposure (Klausz et al., 2011).

Previous studies have proposed a decrease in body weight following prenatal opioid exposure to be a result of intrauterine growth restriction (IUGR) (Devarapalli et al., 2015). However, in our study, a decrease in body weight was only observed in male pups during the early postnatal period (PND 0- PND 7) (Stevens & Mohan, 2021). Thus, it is unlikely that this sustained decrease in body weight is a result of IUGR, as the decreased body weight was more pronounced in adult female offspring. It is unclear why a decrease in body weight was observed into adulthood in morphine exposed spiny mice. However, one study found a decrease in stress marker organs such as the thymus, spleen and adrenal gland as well as a decrease in body weight following prenatal opioid exposure. These changes were associated with hypoactivity of the HPA axis and an increase in depression-like behaviors (Klausz et al., 2011). It is possible that the reduced body weight observed in morphine exposed spiny mice may be indicative of depressive-like behaviors however, further studies are needed.

CONCLUSION

In summary, our findings suggest mid to late gestational opioid exposure results in deficits in short term memory that persist from adolescence into adulthood in spiny mice offspring. Our findings are consistent with previous research which found that prenatal opioid exposure led to impaired learning and memory and development. We observed a decrease in spatial working memory and spatial recognition memory in offspring chronically exposed to morphine. Differences in memory were observed between morphine and saline exposed offspring, which persisted from adolescence to adulthood. This suggests that infants with NOWS may be at risk of long-term deficits in learning and memory. This study is the first to examine the long-term effects of prenatal morphine exposure in spiny mice, a unique rodent model of NOWS. In addition to the withdrawal behavior previously reported, these findings further support the use of this unique rodent species as a more clinically translatable model to understand both the short- and long-term implications of NOWS on behavior and neurodevelopment. Based on previous research, it is likely that prenatal morphine exposure results in impaired development of the hippocampus in spiny mice. However, future studies are needed to understand the underlying molecular changes associated with the impaired memory exhibited in adult spiny mice following prenatal opioid exposure.

CHAPTER 6

GENDER DIFFERENCES IN WITHDRAWING INFANTS

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ABSTRACT

Objective. To assess gender differences in infants diagnosed with neonatal abstinence syndrome at the Cabell Huntington Hospital in Huntington, West Virginia. *Methods*. This is a single-site retrospective chart review involving 97 infants born treated for neonatal abstinence syndrome at the Cabell Huntington Hospital between April and December 2015. Data were obtained from electronic medical records using a secure online survey tool designed using Qualtrics. Maternal demographics and drug screenings were collected. Infant information was collected for the first 7 days of life including withdrawal symptoms, treatment, and growth parameters. These data were analyzed based on gender, male (N = 62) and female (N = 35), to assess any gender differences among the infants. *Results*. No significant differences were found regarding birth weight, length, and gestational age between male and female infants. Differences among the percentage of symptoms experienced were found with females experiencing a greater percentage of symptoms affecting the autonomic nervous system compared with males. Significant differences in head circumference were found in these infants; females were found to have a greater head circumference at time of birth compared with males ($P = .003$), whereas at time of discharge head circumference was greater in males than in females ($P = .035$). *Conclusion*. Differences in symptoms, physical characteristics, and methadone treatment were found between male and female infants diagnosed with neonatal abstinence syndrome at the Cabell Huntington Hospital during 2015. Further studies are needed to assess both the short- and long-term effects of antenatal drug abuse.

INTRODUCTION

The current state of the nation's substance abuse and addiction epidemic has reached emergency status. However, this status has been in a state of emergency in rural West Virginia for over 5 years. In Cabell County, West Virginia, arguably the epicenter of the state's substance abuse emergency there has been a sharp increase in the prescribing rate for opioid analgesics, rates of opioid overdose (from 146 in 2012 to 944 in 2015), and of overdose deaths from 2012 to 2016 (Patrick, Davis, Lehmann, Lehman, & Cooper, 2015). In addition to the rate of overdoses in Cabell County, there has also been a significant increase in substance abuse among pregnant females that has resulted in an increase in number of infants being diagnosed with neonatal abstinence syndrome (NAS). NAS is a post- natal drug withdrawal syndrome that occurs following in utero exposure to drugs. Nationally, the incidence of maternal opioid use during pregnancy is 5.6/1000 live births. During this time, there was a subsequent 5-fold increase in the incidence of NAS, resulting in an estimated 21 732 infants born in the United States in 2012 (Patrick et al., 2015; Patrick et al., 2012). Using Centers for Disease Control and Prevention reports, West Virginia has the highest rate of babies born with NAS in 2013 at 33.4 per 1000 births (Ko et al., 2016). Infants suffering from NAS are more likely to develop adverse health outcomes including low birth weight, preterm birth, admission to the neonatal intensive care unit (NICU), and may require pharmacological treatment that can lead to an extended hospital stay and higher health care expenses. The average length of stay for an infant with NAS was 16.9 days compared with the average 2.1 day stay for non-NAS infants. This extended length of stay is associated with an estimated average cost between US$66, 700 and US$93, 400 per infant depending on need for treatment for the infants with NAS, compared with the US$3500 for infants without NAS. Medicaid coverage accounted for many hospital charges, with 81.5% of

infants diagnosed with NAS enrolled in state programs during 2009 to 2012 (Patrick et al., 2015).

In infants suffering from NAS, maternal opioid use is the most common cause for this syndrome; however, other drugs such as psychostimulants, selective serotonin reuptake inhibitors, barbiturates, benzodiazepines, and cannabis have also been shown to cause neonatal withdrawal (Hudak, Tan 2012; Ross et al., 2014). There are many factors that influence the onset, severity, and duration of withdrawal symptoms, including duration of exposure, time since last dose, as well as the type, amount, half-life, and ability of the drug to cross the placenta. Polysubstance abuse of drugs, tobacco, and alcohol are also common among substance abusers and can affect the development and degree of withdrawal in exposed infants (Kaltenbach & Jones, 2016).

This postnatal withdrawal syndrome is characterized by a variety of symptoms that typically manifest during the first 48 to 72 hours after birth involving the central nervous, autonomic, and gastrointestinal (GI) systems (Kocherlakota, 2014). Opioids are most commonly seen in infants with NAS, and their action on opioid receptors within the developing fetus is the underlying mechanism for an array of withdrawal symptoms. For example, symptoms might include numerous changes in neurological functions such as tremors, seizures, exaggerated moro reflex, and yawning. Infants with NAS often experience GI disturbances and common symptoms include vomiting, diarrhea, and poor feeding. Fever, sweating, mottling, increased heart rate, and sneezing are symptoms associated with dysregulation of the autonomic nervous system (Kocherlakota, 2014). To monitor the progression of withdrawal symptoms associated with NAS, babies are commonly evaluated using the Finnegan scoring system (Sarkar & Donn, 2006). Developed in 1975, infants are assessed every 3 to 4 hours for signs of withdrawal, and observed

symptoms are quantified and recorded as abstinence scores. These abstinence scores are used to determine severity of withdrawal, need for pharmacological intervention, and guidance for weaning (Finnegan et al., 1975). Morphine and methadone are commonly used to treat infants with increased abstinence scores who need pharmacological intervention.

In this retrospective study, we have collected both maternal and patient data from infants who were diagnosed with NAS at the Cabell Huntington Hospital (CHH) in Huntington, West Virginia, which is Marshall University's major medical teaching institution. Recently, the clinician-academics group treating and monitoring infants with NAS at CHH published that between 2010 and 2015, the number of infants treated for NAS at CHH increased by 219% (Loudin et al., 2017). The goals of our report is to provide details on infants with NAS who were born in 2015, the peak of this region's NAS epidemic during this 5-year period with details on demo- graphics, infants born with NAS physical measurements, average Finnegan scores for the first 7 days of treatment, methadone doses and frequencies, and potential gender differences.

METHODS

Data Collection

This is a single-site, retrospective chart review conducted between April 2015 and December 2015. Before data were collected, institutional review board approval was obtained from Marshall University Institutional Review Board. Data were collected from the electronic medical records used at CHH using a specialized and secure online survey designed using Qualtrics. This survey was designed to collect an extensive list of patient information from the mother and the infant before and during treatment for NAS and was designed to minimize user error during the extraction and collection of data from the electronic medical records used at

CHH. Only infants admitted to the NICU and/or the neonatal therapeutics unit between April 2015 and December 2015 treated with methadone for NAS were collected and analyzed. Details of age, ethnicity, marital status, level of education, insurance status, recipient of prior treatment for substance abuse, hepatitis C status, drug screen results from either urine or umbilical cord, and the type of delivery were collected from each mother who delivered an infant suffering from NAS. For each infant diagnosed with NAS, the following maternal information was collected: prenatal care, polysubstance abuse, and toxicology screen results. Also, the following infant birth parameters were collected: gestational age, day, month and year of birth, weight, length, head circumference (HC) at birth and on discharge, gender, ethnicity, and Finnegan (ie, NAS) scores for up to 7 days following the initial diagnosis of NAS. All data were recorded without identifiers to maintain patient confidentially. For collecting scores and recording withdrawal, the standard, most commonly used 21-point Finnegan scoring method was used. We recorded the initial day of scoring following birth up until the seventh day of life. The day and time of the highest Finnegan score was also recorded. The Finnegan scoring method is divided into systems: central nervous system, GI, respiratory, and autonomic disturbances. The following were assessed for central nervous system disturbances: crying, sleep, reflexes (eg, hyperactive Moro reflex), tremors, muscle tone, myoclonic jerking, convulsions, and excoriation. The following were assessed for GI disturbances: excessive sucking, poor feeding, projectile vomiting, and loose and watery stools. For respiratory system disturbances, nasal flaring and respiratory rate with and without retractions were recorded; and last, for disturbances in the autonomic system, the following were recorded: sweating, fever, frequency of yawning, mottling, nasal stuffiness, and sneezing were recorded for each infant diagnosed with NAS.

Regarding data on methadone treatment, the methadone protocol initiation date and time following birth was recorded as well as the termination date and time. Also, the frequency and doses of methadone administered at each step of the treatment protocol as well as the total (mg) amount of methadone administered were all recorded for up to 7 days following initiation of the methadone treatment. We also collected the in-utero exposure information from what was either self-reported (maternal history) or provided from urine and/or umbilical cord toxicological reports either during pregnancy or at time of delivery. Inclusion criteria included patients initiated on methadone between April 2015 and December 2015 admitted to the NICU and/or neonatal therapeutics unit at CHH. Exclusion criteria included patients started on methadone prior to admission at CHH and infants transferred to or from another facility during treatment. The primary outcome was to determine the differences in NAS symptoms and scores between male and female infants receiving oral methadone. Also, total amount of methadone (mg/kg), time (hours) to recording the first NAS score, peak NAS score within the 7-day period following birth, and the peak NAS score during the first 24 hours of life were recorded and com- pared between genders. Patient information included birth weight (kg), length (cm), HC (cm), gestational age (weeks), and the duration of treatment (days).

Data Analysis

Infants were divided into 2 groups for analysis based on their gender, male (N = 62) or female (N = 35). Mean data and percentage (%) difference between males and females with NAS symptoms were calculated and are presented in Tables 4.1 and 4.2. Statistical analysis was run on the data using the Student's unpaired *t* test. $P < .05$ was considered statistically significant.

RESULTS

Patient Characteristics and Methadone Initiation and Dosing

Ninety-seven infants met the inclusion criteria for this study. Therefore, we recorded data from 62 male and 35 female infants diagnosed with NAS; however, for some of the parameters, we could only collect data from less patients as some data points were missing. There were no differences in the baseline characteristics between male and female infants. For example, birth weight, length, gestational age and total duration of treatment were not significantly different between genders. There were no differences in the mean NAS score throughout treatment for oral methadone between male and female infants (data not reported). However, there were differences in the percentage of scores recorded for specific individual NAS symptoms used to record Finnegan scores. We found that female infants suffered from diarrhea, excessive sucking, tremors when disturbed, sleep disturbances, and crying significantly greater than male infants. However, male infants had significantly greater hyperactive Moro reflex and nasal stuffiness compared with female infants.

	Gender	N	Mean	Standard Deviation	*P*
Birth					
Weight (kg)	Male	61	2.90 ± 0.05	0.464	.930
	Female	35	2.89 ± 0.08	0.488	
Length (cm)	Male	61	48.46 ± 0.33	2.60	.944
	Female	35	48.50 ± 0.38	2.29	
Head circumference (cm)	Male	61	33.58 ± 0.19	1.53	.003*
	Female	25	34.74 ± 0.35	1.79	
Discharge					
Weight (kg)	Male	60	3.55 ± 0.09	0.72	.395
	Female	33	3.421 ± 0.11	0.67	
Length (cm)	Male	51	52.55 ± 0.61	4.38	.412
	Female	28	51.78 ± 0.56	2.99	
Head circumference (HC) (cm)	Male	47	35.78 ± 0.27	1.87	.035*
	Female	25	33.40 ± 1.43	7.18	
Gestational age (weeks)	Male	62	37.97 ± 0.19	1.57	.995
	Female	35	37.97 ± 0.28	1.66	
Duration of treatment (days)	Male	58	31.02 ± 1.99	15.2	.858
	Female	33	31.41 ± 1.78	10.24	

*$P < .05$ indicates statistically significant.

Table 6.1. Comparison of neonatal information data at birth and discharge between genders.
Abbreviations: NAS, neonatal abstinence syndrome; GI, gastrointestinal; ANS, autonomic nervous system; CNS, central nervous system

	Gender	N	Percentage (%)	Mean	Standard Deviation	*P*
GI	Male	62	15.21		—	
	Female	35	21.51		—	
ANS	Male	62	20.42		—	
	Female	35	67.35		—	
CNS	Male	62	67.35		—	
	Female	35	64.41		—	
Peak NAS score 24 hours postbirth	Male	52	—	6.28 ± 1.03	7.48	.192
	Female	29	—	9.03 ± 2.09	11.28	
Peak NAS score during 7-day period	Male	52	—	14.96 ± 0.38	2.80	.543
	Female	29	—	15.34 ± 0.46	2.52	
Hours to recording first NAS score	Male	62	—	8.92 ± 1.24	9.81	.167
	Female	35	—	6.44 ± 0.86	5.14	
Methadone total (mg/kg) over 7-day period	Male	24	—	3.58 ± 0.65	3.22	.836
	Female	9	—	3.32 ± 1.06	3.32	
Hours to first methadone dose	Male	62	—	56.63 ± 5.49	32.52	.324
	Female	35	—	49.76 ± 4.19	32.99	

Table 6.2. Comparison of NAS symptoms, scores, and methadone dosing (mg/kg) between genders.
Abbreviations: NAS, neonatal abstinence syndrome; GI, gastrointestinal; ANS, autonomic nervous system; CNS, central nervous system

We found that female (N = 9) infants had less total methadone administered at 3.32 mg/kg compared with male (N = 29) infants at 3.35 mg/kg during the 7-day post- birth period assessed. This difference in total methadone administered (mg/kg) may indicate that male infants may have needed greater methadone before NAS scores reduced, and thus methadone levels adjusted accordingly. Another important parameter we analyzed was the number of hours before the first dose of methadone was administered. We have found that it took an average 56.63 ± 5.49 hours before male infants received their first dose of methadone following diagnosis with NAS. For female infants, it took 49.76 ± 4.19 hours before they received their first dose of methadone. Head circumference (HC) (cm) at birth and discharge was significantly different; at birth, the HC was greater in female infants compared with male infants (P = .003), but at discharge, the HC of the male infants was greater when compared with the female infants (P = .035).

DISCUSSION

Our retrospective review of 97 (62 males and 35 females) infants did not show differences in weight gain, length, and HC between genders. Differences were detected in the percentage of scores recorded for specific individual NAS symptoms. We found that female infants suffered from diarrhea, excessive sucking, tremors when disturbed, sleep disturbances, and crying greater than male infants. We also found differences for methadone initiation time (hours) and duration (days) of methadone between genders. Male infants had a greater methadone initiation time compared with female infants, but this time was comparably the same for both genders before the first dose of methadone was administered. Female infants also received less methadone (mg/kg) than male infants during the 7-day treatment period assessed.

A significant limitation of this study is the small sample size, as it may limit the generalization of the findings. Another limitation is that the oral methadone with its long plasma half-life of 16 to 25 hours in neonates (gestational age 34-43 weeks) may have limited the initial control of NAS symptoms and thus delayed weaning (Rosen & Pippenger, 1976). However, conflicting results have been reported that show that the mean elimination half-life of 41 hours, which indicates that neonates have slower plasma clearance of methadone (Mack, Thomas, Giles, & Buchanan, 1991). Both studies, however, were complicated by unreported maternal ingestion of methadone, exposure to other drugs of abuse during pregnancy, and variable intervals between the last dose of methadone and delivery. The greatest difference between male and female genders was in the type of NAS symptoms experienced over the 7-day period of this study. The differences in NAS symptoms could be due to several factors. For example, the difference in sample size, the type of drugs being taken by the mothers during pregnancy, and at what stage of pregnancy could all affect the symptoms experienced by the infants. The most interesting finding regarding differences in NAS symptoms was that the female infants had 67.35% of their symptoms associated with the autonomic nervous system compared with 20.42% for the male infants. For example, we found that the female infants had greater excessive sucking, tremors when disturbed, sleep disturbances, and crying. Furthermore, detailed studies are warranted to elucidate the impact of polysubstance abuse on symptoms associated with NAS as well as the impact of currently Food and Drug Administration–approved treatments used to treat pregnant females with substance abuse disorders such as Suboxone on NAS symptoms.

CONCLUSION

More studies are needed to assess the short- and long-term effects of antenatal drug abuse. Future studies would include developmental follow-up assessments of the child at different ages and compare these records with the type of opioid (e.g., methadone vs morphine vs buprenorphine) administered to the child to control their NAS symptoms at birth. A more detailed understanding of the short-term (<5 years old) and long-term (5-18 years old) developmental effects of NAS are much needed; however, we also need to consider the effects of treatment on these children.

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Authors' Note

Any underlying research materials used to collect these data can be accessed through email to the corresponding author

Author Contributions

SS: Contributed to conception and design; contributed to analysis; drafted the manuscript; critically revised the manuscript; gave final approval.

TF: Contributed to conception and design; contributed to analysis; drafted the manuscript.

KH: Contributed to analysis.

CT: Contributed to conception and design; contributed to analysis.

JC: Contributed to analysis.

SM: Contributed to conception and design; contributed to analysis; drafted the manuscript; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

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CHAPTER 7

DISCUSSION

Summary

The goal of this dissertation was to develop a more translational rodent model to further our understanding of the effects of prenatal opioid exposure on the developing fetus. As mentioned earlier, current animal models of prenatal opioid exposure are limited by short gestational periods, large litter sizes, and primary organogenesis occurring postnatally. These limitations present the need for a more suitable animal model to study the effects of prenatal opioid exposure.

The first aim of this study was to characterize symptoms of opioid withdrawal in offspring prenatally exposed to morphine using a novel rodent species, *Acomys cahirinus* (spiny mice). We chose to use spiny mice because of their unique biological characteristics that make them an ideal species for perinatal research. We found that chronic maternal morphine exposure led to withdrawal behaviors in spiny mice pups that were previously characterized in other neonatal rodent models of prenatal opioid exposure such as face cleaning, wall climbing, ultrasonic vocalizations, and tremors. However, spiny mice are a precocial species, and their advanced development at birth allowed us to also assess jumping and wet dog shakes which are withdrawal behaviors that are not observed until adolescence or adulthood in other rodent models. Our findings validate the use of spiny mice as a novel model for prenatal opioid exposure.

The second aim of this study was to assess the long-term consequences of prenatal morphine exposure on learning and memory. Offspring prenatally exposed to morphine were found to have deficits in both spatial reference and recognition memory. These deficits persisted

from adolescence to adulthood in morphine exposed offspring. In addition to cognitive deficits, these mice were found to have lower body weights compared to saline exposed offspring. These findings suggest that prenatal opioid exposure has a lasting impact on cognition and somatic growth that persists from adolescence into adulthood.

Ultimately, the goal of our research is to better understand the clinical implications of prenatal opioid exposure on the developing fetus. A retrospective chart study was conducted to collect information regarding withdrawal symptoms, treatment, and growth parameters of infants diagnosed with NAS at Cabell Huntington Hospital. This study revealed that an overall greater number of males were diagnosed with NAS from April 2015 to December 2015. Additionally, gender differences were found between the timing of initiation of methadone treatment, total methadone administration, and the withdrawal symptoms experienced by male and female infants (Stevens et al., 2018).

Gender Differences

Among all three studies there was one major commonality, gender differences. We observed gender differences in the withdrawal behavior, learning and memory, and somatic growth in spiny mice following in utero opioid exposure. Similarly, our retrospective chart study revealed several gender differences among male and female infants who were diagnosed with NAS. Interestingly, there were some similarities observed between our preclinical model and the data we found in our clinical study.

Gender differences were observed in several of the withdrawal behaviors assessed in spiny mice. Similarly, differences in withdrawal symptoms were also observed in male and female infants diagnosed with NAS. More specifically, 67.35% of symptoms in female infants

were associated with the autonomic nervous system compared 20.42% in males. These findings suggest that prenatal opioid exposure effects the developing fetus differently among male and female offspring, leading to different withdrawal behaviors postnatally.

Additionally, differences in growth parameters were observed in both studies. Males prenatally exposed to morphine were found to have a significantly lower body weight compared to saline exposed offspring during the early postnatal period (PND 0 – 7). This decrease in body weight was not observed in females. Lower body weights were observed in both male and female spiny mice prenatally exposed to morphine from 1.5 - 3.0 months of age. However, the difference in body weight was more pronounced in female offspring. Similarly, male infants with NAS were found to have a smaller head circumference at birth compared to females. In contrast, females were found to have a smaller head circumference compared to males at time of discharge. These findings suggest that prenatal morphine exposure may have a greater effect on somatic growth in males during the early postnatal period, and these effects are more pronounced in females later in life.

Collectively these studies provide evidence that prenatal opioid exposure impacts male and female offspring differently. Several studies reported gender specific effects on neurotransmitter systems in offspring following prenatal opioid exposure. These include changes in levels of norepinephrine (NE), acetylcholine (ACh), and 5-HIAA as well as the turnover rates of NE, ACh, and dopamine (DA) (Byrnes & Vassoler, 2017). Gender specific effects on neurotransmitter development may explain the differences observed among male and female offspring following prenatal opioid exposure. Neuronal activation of the noradrenergic system is a known mediator in the expression of symptoms associated with opioid withdrawal both neonates and adults (Ceger & Kuhn, 2000; Maldonado, 1997; Nestler & Aghajanian, 1997). The

abrupt discontinuation of opioid exposure that occurs at birth leads to an excessive release of NE. The released NE binds to noradrenergic receptors which in turn leads to an overactivation of the autonomic nervous system (Stevens, Heffner, Flaugher, & Mohan, 2017). Differences in hypothalamic NE levels and NE turnover were observed in male and female offspring following prenatal opioid exposure. Males were found to have a greater level of NE within the hypothalamus as well as an increase in NE turnover rate. In contrast, a decrease in hypothalamic NE levels and turnover were observed in female offspring (Robinson et al., 1997; Vathy et al., 1994). Additionally, ACh is implicated in the development of several withdrawal symptoms observed in withdrawing infants (Kocherlakota, 2014). A decrease in striatal ACh levels were observed in both male and female offspring following prenatal methadone exposure, however this decrease was more pronounced during the early postnatal period in females (Robinson, 2000, 2002; Robinson, Guo, Maher, McDowell, & Kunko, 1996; Robinson, Mo, Maher, Wallace, & Kunko, 1996). Interestingly, striatal levels of ACh were found to be greater in females than males during the second and third postnatal week (Robinson, 2002). Collectively, these gender specific differences in postnatal NE and ACh levels and turnover rate may account for some of the observed differences in withdrawal behavior in male and female offspring.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

Our studies demonstrated the use *Acomys cahirinus* in a novel rodent model of NOWS. This translational model will be the basis for our future studies to elucidate the cellular mediators associated with these short and long-term effects of prenatal opioid exposure. More specifically, we are interested in understanding the involvement of glial cells in the development of neurotoxicity in the developing fetal brain induced by maternal opioid administration.

Astrocytes and microglia are implicated in numerous brain functions including synaptic development and maturation, synaptic plasticity, neurotransmitter release and uptake, neuronal cell survival, neurodevelopment, and initiating an immune response (Bilbo & Schwarz, 2012; Eroglu & Barres, 2010; Kettenmann, Kirchhoff, & Verkhratsky, 2013; Salter & Beggs, 2014; Sofroniew & Vinters, 2010). Studies have shown that drugs of abuse, including opioids, can lead to altered glial activation and function within the adult models of exposure (Bachtell, Jones, Heinzerling, Beardsley, & Comer, 2017; Coller & Hutchinson, 2012; Miguel-Hidalgo, 2009). It has also been suggested that glia are implicated in the progression and manifestation of drug addiction (Lacagnina, Rivera, & Bilbo, 2017). However, little is known about the effects of opioid exposure on glial maturation, activation, and function during embryonic development.

We hypothesized that prenatal morphine exposure induces glial activation resulting in an exacerbated neuroinflammatory response which results in altered brain development. A recent study which demonstrated activation of microglia and a heightened immune response following late gestation methadone exposure validated our hypothesis. However, this study chose postnatal drug exposure in a rat model (Jantzie et al., 2020). The limitations of this method of modeling NOWS have been previously described. Using our spiny mice model, we plan to investigate the

effects of prenatal opioid exposure on astrocytes and microglia during the early postnatal period, adolescence and adulthood. Additionally, we are interested in investigating any gender specific differences in glial activation. We are hopeful that these studies will expand our understanding of the effects of prenatal opioid exposure and also reveal novel targets to develop preventative treatment strategies for NOWS.

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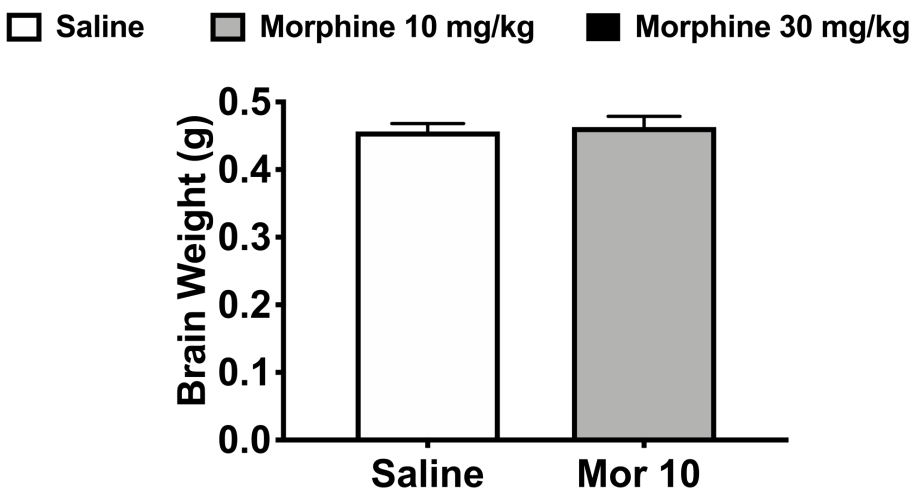
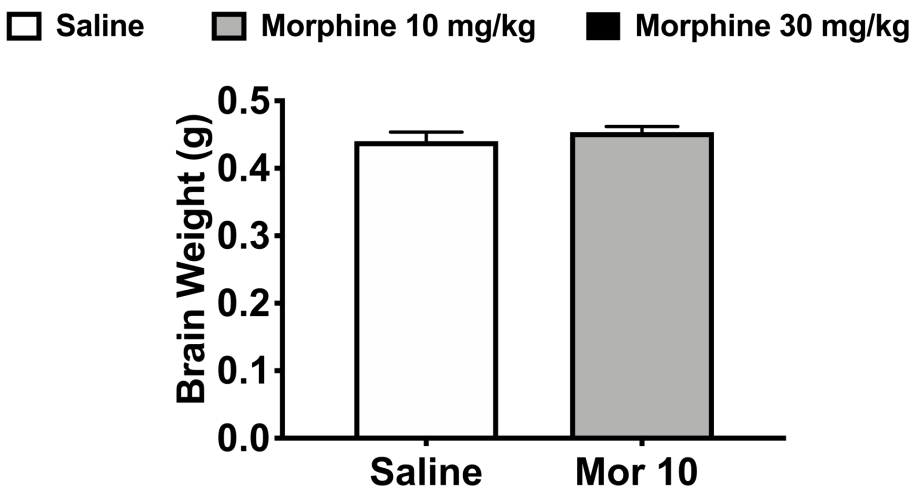
APPENDIX A

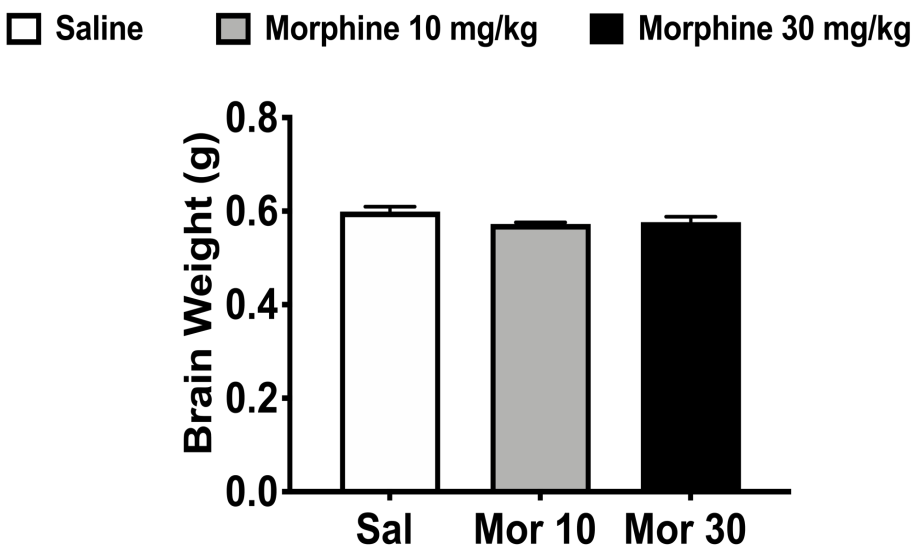
SUPPLEMENTARY FIGURES



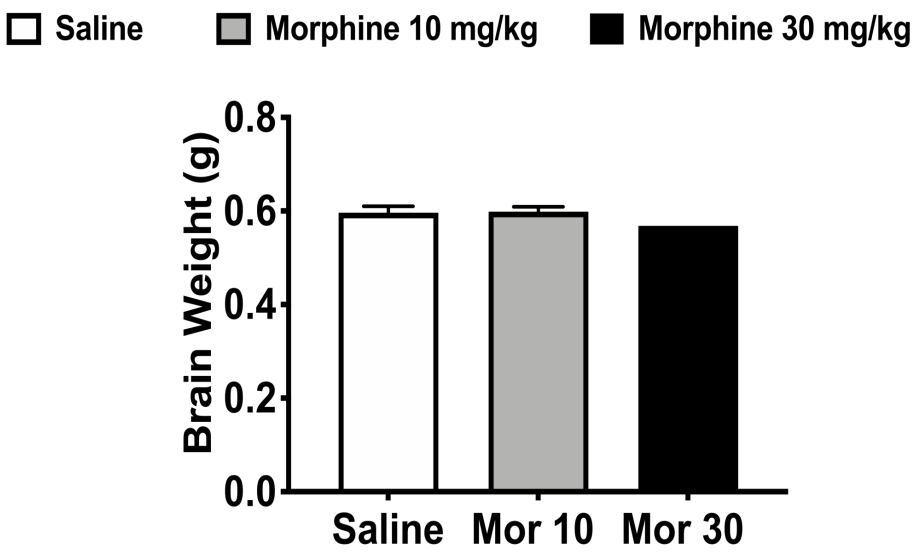
Supplementary Fig. 1 Male Brain Weight on Postnatal Day 0
On PND 0 there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=4) and saline-treated controls (N=6) in male spiny mice. Data are represented as mean totals (± SEM).



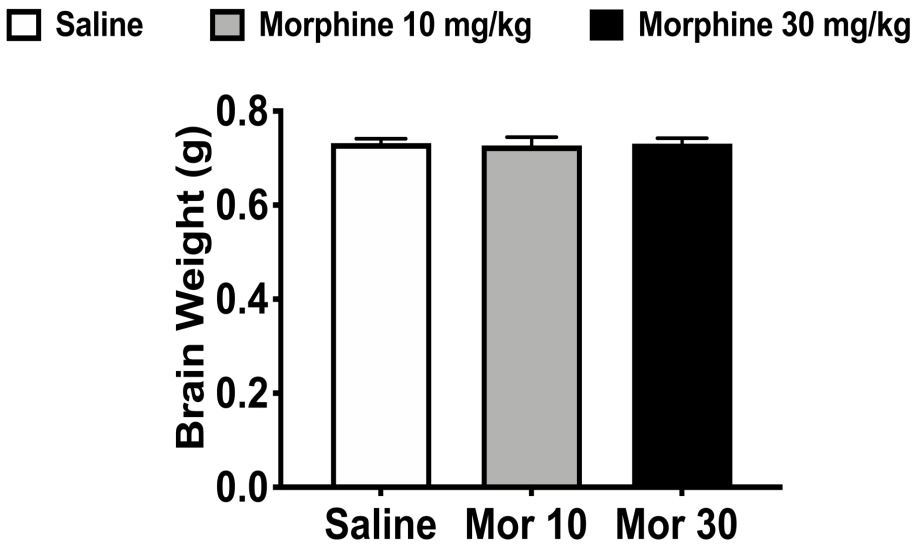
Supplementary Fig. 2 Female Brain Weight on Postnatal Day 0
On PND 0 there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=7) and saline- exposed controls (N=4) in male spiny mice. Data are represented as mean totals (± SEM).



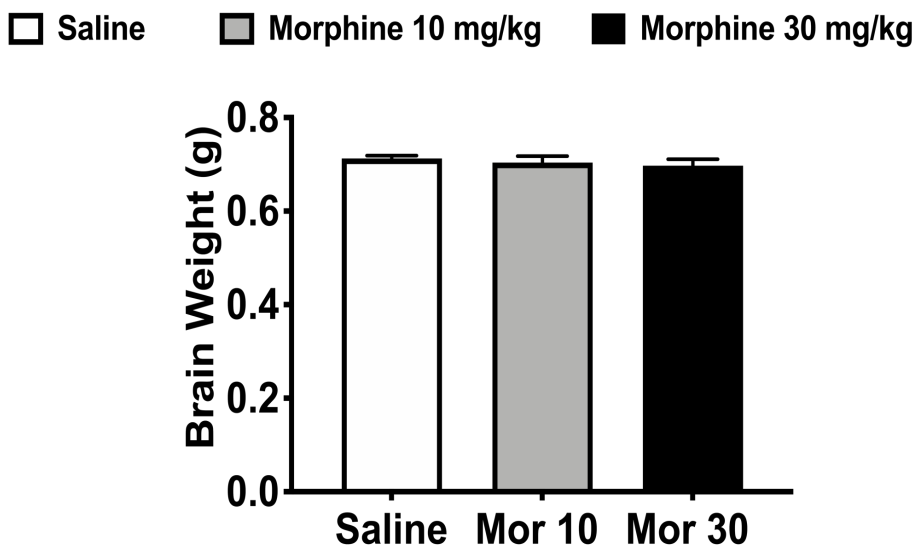
Supplementary Fig. 3 Male Brain Weight on Postnatal Day 7
On PND 7 there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=6, 30 mg/kg: N=2) and saline- exposed controls (N=6) in male spiny mice. Data are represented as mean totals (± SEM).



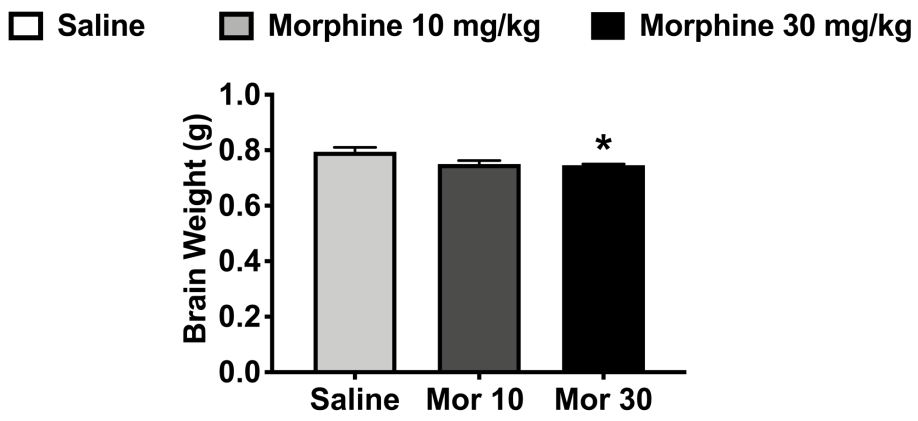
Supplementary Fig. 4 Female Brain Weight on Postnatal Day 7
On PND7 there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=6, 30 mg/kg: N=1) and saline- exposed controls (N=5) in female spiny mice. Data are represented as mean totals (± SEM).



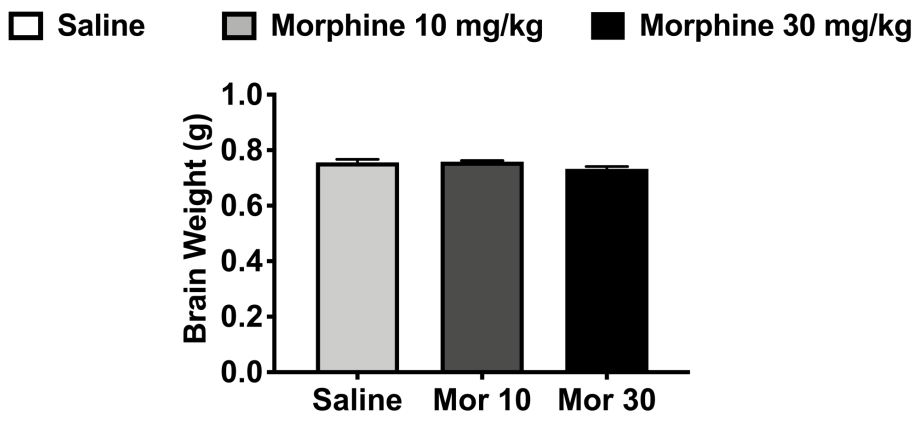
Supplementary Fig. 5 Male Brain Weight at 1 Month of Age
At 1 month of age there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=6, 30 mg/kg: N=6) and saline- exposed controls (N=6) in male spiny mice. Data are represented as mean totals (± SEM).



Supplementary Fig. 6 Female Brain Weight at 1 Month of Age
At 1 month of age there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=6, 30 mg/kg: N=6) and saline-exposed controls (N=6) in female spiny mice. Data are represented as mean totals (± SEM).



Supplementary Fig. 7 Male Brain Weight at 3 Months of Age
At 3 months of age 30 mg/kg morphine exposed mice (N=6) had a significantly lower brain weight (g) compared to saline-exposed controls (N=6) in male spiny mice. Data are represented as mean totals (± SEM). An asterisk symbol (* vs. saline) is used to denote significance between exposure groups, $P < 0.5$.



Supplementary Fig. 8 Female Brain Weight at 3 Months of Age
At 3 months of age there was no significant difference in brain weight (g) among morphine exposed mice (10 mg/kg: N=6, 30 mg/kg: N=6) and saline-exposed controls (N=6) in female spiny mice. Data are represented as mean totals (± SEM).

APPENDIX B

IACUC APPROVAL LETTER

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Animal Resource Facility

DATE:	June 7, 2017
TO:	SHEKHER MOHAN, Ph.D.
FROM:	Marshall University IACUC
IACUC #:	681
PROJECT TITLE:	[1048744-5] Measuring gender differences in neurodevelopment using a mouse model of opioid-induced neonatal abstinence syndrome (NAS)
SUBMISSION TYPE:	Amendment/Modification
ACTION:	APPROVED
APPROVAL DATE:	June 7, 2017
EXPIRATION DATE:	April 30, 2020
REVIEW TYPE:	Full Committee Review

Thank you for your submission of Amendment/Modification materials for this research project. The addendum underwent Full Committee Review and was APPROVED.

All research must be conducted in accordance with this approved submission.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC

PURDUE UNIVERSITY

Purdue Animal Care and Use Committee

To:	MUSTAFA, AHMED MOHAN, SHEKHER
From:	Lori Bugher, PACUC Administrative Assistant
Date:	05 / 09 / 2018
Committee Action:	Designated Member Approval
Submission Type:	PACUC Requested Revisions
Approval Date:	05 / 09 / 2018
Protocol Number:	1712001654
Study Title:	Measuring gender differences in neurodevelopment using a mouse model of opioid-induced neonatal abstinence syndrome (NAS)
Expiration Date:	02 / 11 / 2021

Your submission was reviewed and approved by the Purdue Animal Care and Use Committee(PACUC) via designated member review. The submission was approved as presented.

The PACUC office will no longer be mailing out copies of approved protocols since they are available online.

- This is the approval of amendment 1712001654A001.

This approval will remain in effect until: Feb 11, 2021

(This is an automated message; there is no need to respond unless you have a question or problem).

Best Regards,
PACUC Staff.

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April 20, 2016

Leesa Prunty, Pharm.D.
Cabell Huntington Hospital Pharmacy

RE: IRBNet ID# 848647-1
At: Marshall University Institutional Review Board #1 (Medical)

Dear Dr. Prunty:

Protocol Title:	[848647-1] Retrospective review of methadone initiation thresholds in infants with Neonatal Abstinence Syndrome	
Expiration Date:	April 20, 2017	
Site Location:	CHH	
Submission Type:	New Project	APPROVED
Review Type:	Expedited Review	

In accordance with 45CFR46.110(a)(5), the above study was granted Expedited approval today by the Marshall University Institutional Review Board #1 (Medical) Chair for the period of 12 months. The approval will expire April 20, 2017. A continuing review request for this study must be submitted no later than 30 days prior to the expiration date. The approval also includes the Waiver of Informed Consent and the HIPAA Waiver.

If you have any questions, please contact the Marshall University Institutional Review Board #1 (Medical) Coordinator Trula Stanley,MA, CIC at (304) 696-7320 or stanley@marshall.edu. Please include your study title and reference number in all correspondence with this office.

APPENDIX D

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Fig. 2.1 Major neurodevelopmental events across species

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Fig. 2.2 The brain growth spurts of 7 mammalian species expressed at first-order velocity curves of the increase in weight with age

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Fig. 3.1 Phylogenetic tree demonstrating evolution of spiny mouse (red) in relation to other murids

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Fig. 3.2 Postnatal development of *Acomys cahirinus*

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Fig. 3.3 In utero development of *Acomys cahirinus* fetus

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Fig. 3.4 Comparison of newborn *Acomys cahirinus* and rat

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Fig. 3.5 Comparing brain growth between species

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CIRRICULUM VITAE

Sarah Stevens
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EDUCATION

Ph.D. Candidate — Biomedical Science (Graduation: Spring 2021) **2015- Present**
Marshall Univ., School of Medicine, Huntington WV
Advisor: Dr. S. Mohan Ph.D., Assistant Professor, Manchester Univ., College of Pharmacy

Bachelor of Science — Molecular and Cellular Biology (Class of 2012) **2008-2012**
Cedarville Univ., College of Science, Cedarville, OH
Minor: Chemistry and Biblical Studies

PROFFESSIONAL EXPERIENCE

The Pointe Church — Digital Connections Specialist **2020-Present**
Oversee online church services and maintain social media accounts

Ivy Tech Community College — Adjunct Professor **2019-Present**
Anatomy & Physiology w/ lab and Introduction to Biology w/ lab
*Pre-health sciences majors and pre-nursing majors **online and in-class***

Purdue University Fort Wayne — Animal Care Technician **2019-2020**
Maintain animal facilities and provide day to day animal care

Manchester Univ., College of Pharmacy — Teaching assistant **2018-2019**
Pharmacogenomics — Human Genetics and Analytical Tech Lab
Master's Students in Pharmacogenomics — online and in class

Marshall Univ., College of Pharmacy — Teaching assistant **Spring 2018**
Integrated Pharmacy Lab
Pharmacy (Pharm.D.) Students

MountWest Community and Technical College — Adjunct Professor **Fall 2017**
Introduction to Anatomy & Physiology
Pre-health sciences majors and pre-nursing majors

Marshall Univ., School of Medicine — Research Technician **2013-2015**
Conducted obesity and cardiovascular disease research
Managed lab staff, lab ordering, manuscript submissions, and IRB protocols

Cedarville Univ., Dept., of Math and Science — Biology Lab Assistant **2012**
Prepared and set up lab experiments, maintained lab supplies and spaces
Graded students' exams and lab assignments

Cedarville Univ., College of Science — Student Researcher **2010**
Supervisor: Dr. H. Kuruvilla Ph.D.
Conducted research investigating nociception in *Tetrahymena thermophila*

Cedarville Univ., Cove Tutoring Services — Student Tutor **2010**
Instructed students in Biology and Microbiology

ACADEMIC ACHIEVEMENTS AND AWARDS

Best Oral Presentation (Basic Science Category) **March 2017**
Marshall Univ., School of Medicine 27th Annual Research Day

1st Place Poster Presentation (Immunology Category) **Oct. 2016**
Appalachian Regional Cell Conference (ARCC)

Best Academic Performance for 1st Year Ph.D. BMS Student **Aug. 2016**
Marshal Univ., School of Medicine Biomedical Science Program

PROFESSIONAL SKILL SETS

TEACHING

- Instructional and Teaching Platforms – Blackboard® and Canvas®
- Virtual Teaching Certification—Ivy Tech Community College, Fort Wayne, IN
- PowerPoint®, Word®, Excel®
- Wet-labs design and instruction
- Online-virtual lab design and instruction
- Online teaching via Zoom®
- Assessment methods used—paper and lockdown browser with Canvas and Blackboard

RESEARCH

- Wet-lab techniques—protein extraction, Western blotting, ELISA, PCR, Cell Culture, Immunohistochemistry, Immunocytochemistry, cryostat and microtome tissue sectioning, microscopy
- Animal techniques and procedures—mouse handling, S.C. and I.P. injections, dissection, tissue processing and procurement, animal breeding, housing, and behavioral assays to measure opioid withdrawal and learning and memory
- Data analysis software—Excel® and Prism®
- IRB and IACUC protocol writing, editing, and submissions
- Manuscript writing, editing and submitting
- Professional Membership - Society for Neuroscience (SfN) (2017 – Present)

RESEARCH INTERESTS

Neonatal opioid withdrawal syndrome (NOWS) - neurobehavioral and developmental changes induced by prenatal opioid exposure.

PEER-REVIEWED PUBLICATIONS

GRADUATE SCHOOL PUBLICATIONS (2016 – Present)

1. **Stevens. S**., Mohan. S. Opioid Withdrawal Behavior In Spiny Mice: A Novel Preclinical Model of Neonatal Opioid Withdrawal Syndrome (NAS). *Heliyon* (*Under Review*). **2021**
2. **Stevens. S.**, Flaugher T, Hughes K, Terwilliger C, Copley J, Mohan S. Gender Differences in Withdrawing Infants. *Global Pediatric Health*, **2018**
3. **Stevens. S.**, Heffner C, Flaugher T, Mohan S. Neonatal Abstinence Syndrome (NAS): Neurodevelopmental Challenges, Current Treatments and Future Directions. *Current Opinions in Neurological Science***, 2017**
4. Mohan S, Robinson T, Allen N, Armstrong L, **Stevens S**. Morphine-Mediated Cytoprotection against Hemin in SK-N-SH and A172 Cells. *Neurochemistry & Neuropharmacology*, **2016**

PUBLICATIONS (2015 – 2016)

5. Sodhi K, Hilgefort J, Gilliam C, Banks G, **Stevens S**, Ansinelli H, Getty M, Abraham NG, Shapiro J, Khitan Z. Uric Acid-Induced Adipocyte Dysfunction is Attenuated by HO-1 Upregulation: Potential Role of Antioxidant Therapy to Target Obesity. *Stem Cells International,* **2016**
6. Sodhi K, Puri N, Favero G, **Stevens S**, Meadows C, Abraham NG, Rezzani R, Ansinelli H, Lebovics E, and Shapiro J. Fructose Mediated Non-alcoholic Fatty Liver is Attenuated by HO-1-SIRT1 Module in Murine Hepatocytes and Mice Fed a High Fructose Diet. *PLOS ONE,* **2015**
7. Sodhi K, Pesce P, **Stevens S**, Puri N, Getty M, Rezzani R, Favero G, Fabrizio R, Scardoti D, Shapiro J. Impairment of Heme Oxygenase Expression in Immuno-Suppressed Mice Exacerbates Ischemic Heart Myocyte Cell Death: Reversible by Bilirubin and Restoration of Nitric Oxide. *J. of Cardiology and Therapy,* **2015**

REARCH ABSTRACTS (* Poster, # Oral)

1. ***Stevens S**., Mohan S. Assessing Physiological and Behavioral Changes in Rodents Following In Utero Opioid Exposure. Manchester University Annual Research Symposium**,** North Manchester, IN. April 24, **2020**.**Abstract Accepted, conference canceled COVID-19*
2. ***Stevens S**., Mohan S. Assessing Physiological and Behavioral Changes in Rodents Following In Utero Opioid Exposure. Greater Indiana Society for Neuroscience, Indianapolis, IN. April 3, **2020**.**Abstract Accepted, conference canceled COVID-19*
3. ***Stevens S**., Mohan S. Assessing Physiological and Behavioral Changes in Rodents Following In Utero Opioid Exposure. 2020 Tri-State Opioid Symposium, Cincinnati, OH. March 25, **2020**.**Abstract Accepted, conference canceled COVID-19*

4. ***Stevens S**., Mohan S. Assessing Physiological and Behavioral Changes in Rodents Following In Utero Opioid Exposure. Society for Neuroscience, Chicago, Illinois, October **2019**
5. ***Stevens S**., Mohan S. Assessing Behavioral Changes Following In Utero Opioid Exposure in Rodents. Greater Indiana Society for Neuroscience, Indianapolis, IN. March 22, **2019**
6. ***Stevens S**., Seifert A., Jones C., Mohan S. Cell-by-Cell: Understanding Opioid-Mediated Neonatal Abstinence Syndrome (NAS). Marshall Univ., School of Medicine 30th Annual Research Day, March 30th, **2018**
7. ***Stevens S**., Seifert A., Mohan S. Modeling Opioid-Mediated Neonatal Abstinence Syndrome (NAS) to Improve Our Understanding Challenges in Neurodevelopment. Society for Neuroscience, Washington, D.C. November 15th, **2017**
8. ***Stevens S**., Isaacs H., Claar H., Mohan S. Role of Interleukin-1 Receptor (IL-1R) in Morphine-induced Hyperalgesia Using a Rodent Model of Postoperative Pain Mouse. Univ., of Kentucky, 2017 CCTS Spring Conference, March 30th, **2017**
9. Isaacs H., **#Stevens S**., Claar H., Mohan S. Role of Interleukin-1 Receptor (IL-1R) in Morphine-induced Hyperalgesia Using a Rodent Model of Postoperative Pain Mouse. Marshall Univ., School of Medicine 29th Annual Research Day, March 24th, **2017. 1st Prize**
10. Isaacs H., ***Stevens S**., Claar H., Mohan S. Role of Interleukin-1 Receptor (IL-1R) in a Mouse Model of Incisional Pain. Appalachian Regional Cell Conference, Charleston, West Virginia. October 1st, **2016. 1st Prize**
11. ***Stevens S**., Ansinelli H., Bellnar L., Khitan Z., Abraham NG. Increased Levels of Heme-Oxygenase-1 Rescues Fructose Mediated Adipocyte Dysfunction. Experimental Biology, Boston, MA. March 28th-April 1st, **2015**
12. Sodhi K., Meadows C., ***Stevens S**., Lebovics E., Shapiro J., Abraham NG. Fructose Mediated Non-alcoholic Fatty Liver is Attenuated by HO-1-SIRT1 Module in Murine Hepatocytes and Mice Fed a High Fructose Diet. Experimental Biology, Boston, MA. March 28th-April 1st, **2015**
13. ***#Stevens S**., Shapiro J., Sodhi K. Impairment of Heme Oxygenase Expression in Immuno-Suppressed Mice Exacerbates Ischemic Heart Myocyte Cell Death: Reversible by Bilirubin and Restoration of Nitric Oxide. Marshall Univ., School of Medicine 27th Annual Research Day, March 24th, **2015**
14. Sodhi K., Meadows C., ***Stevens S**., Shapiro J. Fructose Mediated Non-alcoholic Fatty Liver is Attenuated by HO-1-SIRT1 Module in Murine Hepatocytes and Mice Fed a High Fructose Diet. Marshall Univ., School of Medicine 27th Annual Research Day, March 24th, **2015**
15. Sodhi K., Maxwell K., ***Stevens S**., Nichols A., Getty M., Liu J., Shapiro J. Na/K-ATPase Mimetic pNAKtide Peptide Attenuates Adiposity and Metabolic Imbalance in Mice Fed a High-fat Diet by Reprogramming Adipocyte Phenotype. Marshall Univ., School of Medicine 27th Annual Research Day, March 24th, **2015**