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Long-Term Immune Consequences of Perinatal Opioid Exposure

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by

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ABSTRACT

Since 2000, the rate of opioid use and abuse by women of reproductive age has increased exponentially, resulting in a five-fold increase in the number of infants exposed to opioids *in utero*. Perinatal opioid exposure is associated with an increased risk of hospitalization and infection, suggesting that chronic exposure to opioids across gestation negatively impacts immune function. Opioid-induced immunocompromise may be due to a direct inhibitory action on immune cells, or indirectly via multiple central nervous system pathways. Opioids also negatively influence the gut microbiota, which serves as an essential immune stimulator. Remarkably few studies have examined the impact of perinatal opioid exposure (POE) on immune system development and function, or gut microbiota composition, and none have utilized a clinically relevant model that recapitulates opioid use disorder in women of reproductive age. Our studies directly address this gap and investigate the influence of *in utero* opioid exposure on basal immune function and response to an immune stimulator in adult male and female rats. The impact on the gut microbiota was examined as well. Here, we report that the febrile and neuroinflammatory response to an immune stimulator, lipopolysaccharide, is potentiated by perinatal opioid exposure, likely via suppressed baseline immune response, including a reduction in antibody production. We also report that perinatal morphine alters gut microbiota composition and significantly decreases microbial maturity. These changes in gut microbiota composition likely contributed to the observed deficits in immune system function. Together, this suggests that *in utero* opioid exposure influences gut microbiota composition and suppresses immune system development, increasing susceptibility to infection. Further

investigation into the mechanisms whereby opioid exposure compromises immune system development is critical for the identification of potential interventions.

INDEX WORDS: Morphine, Perinatal, Lipopolysaccharide, Immunity, Microglia, Microbiota

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DEDICATION

I dedicate this work to my family, who have always been proud of me.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

1.1 The opioid crisis and neonatal opioid withdrawal syndrome (NOWS)

The United States has existed in a state of nationwide public health emergency since 2017 due to the consequences of the opioid crisis (Department of Health and Human Services, 2017). However, this ongoing emergency actually originated as early as the 1990s, when health professionals increased the number of opioids prescribed in an attempt to correct for the undertreatment of pain (Sarkar and Shojania, 2016). This increase in prescription opioids was largely due to the erroneous belief that those experiencing chronic pain were unlikely to develop an opioid use disorder (OUD) (Volkow and Blanco, 2021). Although opioid dispensing rates peaked in 2012 (81.3 opioid prescriptions per 100 persons), rates remained high at 43.3 per 100 persons in 2020 (Centers for Disease Control, 2021). Unfortunately, the reduction in overall prescription number has not been matched by a decrease in overdose deaths, which reached an all-time high of 28.3 per 100,000 people in 2020 (Hedegaard et al., 2021; Volkow and Blanco, 2021). Remarkably, 3.4% of persons over 12 years of age have misused heroin or prescription pain relievers in the past year by not having a prescription or in ways not directed by their doctor (Substance Abuse and Mental Health Services Administration, 2021).

Of particular concern is opioid use and misuse by women of childbearing age, as it is currently estimated that 3.72% of women aged 16-49 years misused opioids in the past year (U.S. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality, 2021). Similar to the general population of opioid users, 95.2% of women with an opioid use disorder were primarily misusing prescription pain relievers (U.S. Department of Health and Human Services, Substance

Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality, 2021). Opioid use by women of childbearing age creates a potential for their offspring to be exposed to opioids during gestation. Indeed, less than 55% of women with opioid and other substance use disorders use contraception, and instead rely largely on less effective contraceptive methods like condoms (Terplan et al., 2015), often resulting in unplanned pregnancies (Heil et al., 2011). Furthermore, 2.99% of pregnant women reported opioid misuse during the previous year (U.S. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality, 2021). The majority of infants exposed to opioids *in utero* will undergo withdrawal following birth, a condition known as neonatal opioid withdrawal syndrome, or NOWS (Doberczak et al., 1991). Rates of NOWS reached 6.3 per 1000 hospital births in 2020, and in some states, the rates are as high as 43.4 per 1000 hospital births (Healthcare Cost and Utilization Project, 2022). The clinical presentation of NOWS includes tremors, irritability, excessive high-pitched crying, gastrointestinal symptoms, and autonomic dysregulation (Kocherlakota, 2014), and children with NOWS experience a longer (increased median stay length of 9.2 days) and more expensive (average of $14400 more than uncomplicated births) hospital stay (Hirai et al., 2021). Despite the large number of children born each year with NOWS, research has focused primarily on immediate postnatal treatment and prevention; little work has been conducted on the long-term consequences of NOWS. Longitudinal studies past the first years of life do not currently exist (Kocherlakota, 2014), raising concerns about potential deficits later in life. As opioids have been associated with immunosuppression, neuroinflammation, and gut microbiota dysbiosis

(Plein and Rittner, 2018; Vallejo et al., 2004; Wang and Roy, 2017), this raises concerns about the long-term effects of perinatal opioid exposure on immune function.

1.2 Opioid pharmacology and metabolism

Morphine was first isolated from the opium poppy in the early 1800s and became the primary drug for pain relief in the 1850s (Brook et al., 2017). Anti-narcotic drug laws were not introduced in the United States until the 1910s, shortly after morphine's addictive properties were first recognized (Brook et al., 2017). Despite these restrictions, morphine is still considered the "gold standard" and first-choice opioid analgesic by the World Health Organization (Brook et al., 2017). Common drugs of abuse such as codeine and heroin are metabolized to morphine, making a clear understanding of how opioid signal, both centrally and peripherally, critical for the development of strategies to combat abuse.

1.2.1 Morphine pharmacokinetics and pharmacodynamics

Opioids bind to three main types of G protein-coupled receptors: μ, δ, and κ (Friedman and Nabong, 2020). Morphine primarily binds to the μ-opioid receptor, which mediates the antinociceptive and rewarding properties of opioids (Friedman and Nabong, 2020). Approximately 10% of morphine is directly absorbed or excreted from the body without metabolism, with the remaining 90% metabolized via glucuronidation in the liver and central nervous system (CNS) into two active metabolites: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Christrup, 1997). M6G binds to the μ-opioid receptor and synergizes with morphine to produce analgesia (Abbott and Palmour, 1988), whereas M3G instead acts on a receptor of the innate immune system, Toll-like receptor 4 (TLR4), to promote inflammation and oppose the analgesic effects of morphine and M6G (Due et al., 2012).

Increased ratios of M3G:morphine, as seen in female vs. male rats (Baker and Ratka, 2002; South et al., 2009), are associated with decreased antinociceptive effects of morphine (Doyle and Murphy, 2018), suggesting that sex-dependent alterations in glucuronidation ratios are correlated with morphine efficacy and action. Importantly, the pharmacokinetics and pharmacodynamics of morphine are dependent on age, with fetuses and young infants particularly vulnerable to opioid exposure.

1.2.2 Fetal-specific pharmacology and metabolism

Opioids, including morphine, are lipophilic and can easily cross the placenta into the fetal brain through the underdeveloped blood-brain barrier (Kocherlakota, 2014). Preterm infants have underdeveloped opioid clearance systems, leading to higher morphine serum concentrations and increased half-life than full-term infants (Scott et al., 1999). Importantly, even full-term infants have clearance rates only one-third that of older children (Lynn and Slattery, 1987). Young infants, up to approximately one year of age, produce higher level of M3G than M6G, promoting inflammation and a weaker analgesic effect of morphine (Bouwmeester et al., 2004). In adults, approximately 35% of morphine is protein-bound and unavailable for receptor binding, leaving 65% of the drug free to signal via μ-opioid receptor binding (Pacifici, 2016). In neonates, the proportion of protein-bound morphine is only 20%, leaving 80% of morphine free to bind (Pacifici, 2016). This increased free portion is primarily due to lower levels of plasma proteins and decreased affinity of those proteins for morphine (Lu and Rosenbaum, 2014). Together, this results in higher exposure levels and accumulation of morphine in the developing brain (Lu and Rosenbaum, 2014; Pacifici, 2016), and suggests that

infants perinatally exposed to opioids may be even more susceptible than adults to the negative consequences of opioids due to increased accumulation and altered metabolism.

1.3 Opioids and the immune system

Morphine's effects on cellular immunity and susceptibility to infection were first identified in the late 1800s (Vallejo et al., 2004). However, throughout the 1900s, heroin users were considered susceptible to disease due to communal needle use or contaminated drug supply (Hussey and Katz, 1950). Only in the 1990s did a full appreciation of the role of μ-opioid receptors in immune function arise (Vallejo et al., 2004), and it is now well established that morphine has immunosuppressive effects independent of unsafe injection practices (Roy et al., 2011). However, to date, the exact mechanisms by which opioids suppress the immune system have not yet been fully elucidated.

1.3.1 Overview of the immune system and its development

Nearly all immune cells arise during development from hematopoietic stem cells (HSCs) (Abbas et al., 2017) (see Figure 1-1). HSCs differentiate into either the myeloid or lymphoid lineage depending on the type, number, and abundance of cytokines present in the hematopoietic environment during proliferation and differentiation (Abbas et al., 2017). It is the development and differentiation of HSCs that form the basis of the adult immune system.

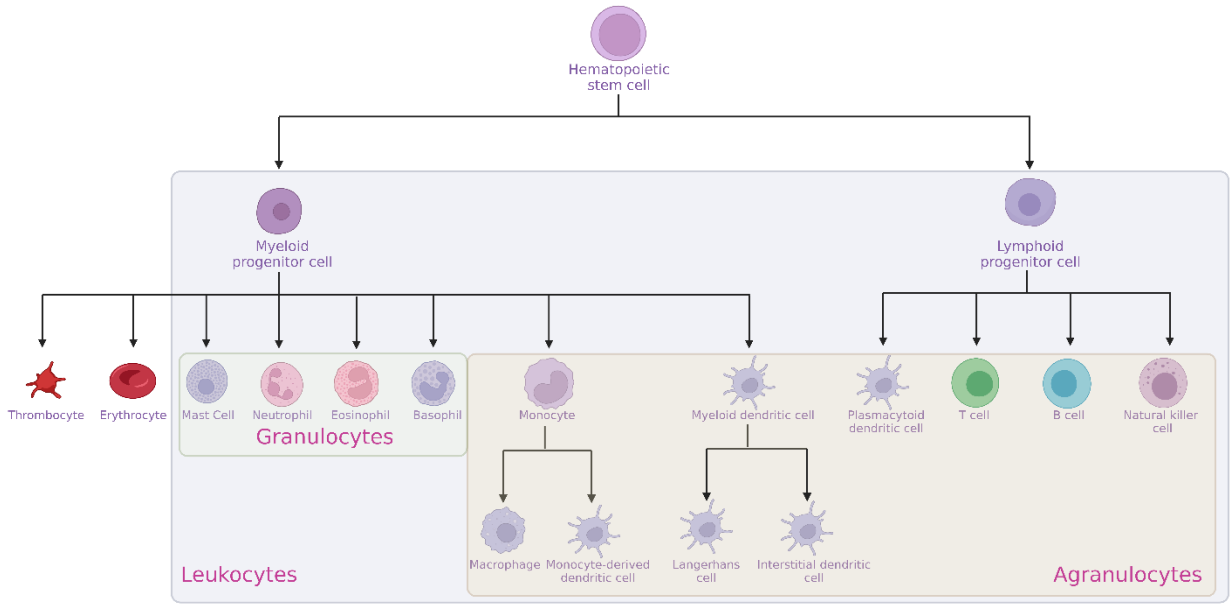


Figure 1-1. Overview of hematopoietic stem cell development and lineage. Created with Biorender.

The immune system's primary role is to respond to foreign material through two main branches: innate immunity and adaptive immunity (Abbas et al., 2017). Innate immunity is the first line of defense against pathogens and relies mainly on physical barriers and non-specific immune system components (e.g., macrophages and natural killer cells). In contrast, adaptive immunity is stimulated by exposure to a specific antigen and is mediated primarily by B and T lymphocytes (Abbas et al., 2017).

1.3.2 Innate immunity

Innate immunity acts predominantly at epithelial surfaces (e.g., the skin and gastrointestinal tract) to prevent microbial colonization by creating physical barriers of epithelial cells connected by tight junctions (Abbas et al., 2017). In this capacity, the innate immune system protects the body until the adaptive immune system is fully engaged, a process

which can take several days (Abbas et al., 2017). The innate immune system responds to pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) through the activation of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (Abbas et al., 2017). The binding of PAMPs and DAMPs to PRRs leads to the activation of transcription factors, including NF-κB and AP-1, to produce proinflammatory cytokines and chemokines (Abbas et al., 2017). Macrophages and natural killer cells are then activated by cytokine release to kill microbes as well as infected or injured cells (Abbas et al., 2017).

A major response of the innate immune system is fever. When PAMPs binds to PRRs, this promotes cytokine release, including TNF-α, IL-1α, IL-1β, and IL-6, which signal to the brain to initiate prostaglandin E2 (PGE2) synthesis in the hypothalamus (Abbas et al., 2017; Evans et al., 2015). PGE2 then binds to EP3 receptors on thermoregulatory neurons in the median preoptic nucleus (MnPO) of the hypothalamus, which then signals downstream to promote vasoconstriction and metabolism of brown adipose tissue to raise body temperature (Evans et al., 2015). This increase in core body temperature then stimulates aspects of both the innate and adaptive immune system, including neutrophil and macrophage activation, natural killer cell cytotoxicity, dendritic cell-mediated phagocytosis, and lymphocyte trafficking and proliferation, and also limits microbial replication (Abbas et al., 2017; Evans et al., 2015). A rapid return to normal body temperature is essential to prevent damage to host cells via prolonged fever or excessive release of cytokines (Evans et al., 2015). Fever is typically accompanied by a set of depressive-like symptoms termed “sickness behavior” (Lasselin et al., 2020). This complex set of behaviors is evolutionarily conserved and includes fatigue, reduced appetite and food consumption, and social withdrawal (Lasselin et al., 2020). Sickness behavior

is theorized to conserve energy to promote immune responses as well as signal the sickness state of the organism to others, preventing transmission of infection (Lasselin, 2021).

1.3.3 Adaptive immunity

Innate immune system activation, especially cytokine release, is also required for the proper adaptive immune system response, including immunological “memory” formation (Abbas et al., 2017). The adaptive immune response begins with the presentation of antigens to B lymphocytes by antigen-presenting cells (APCs) (Abbas et al., 2017). Although the entire population of lymphocytes can respond to millions of diverse antigens, each B cell expresses only one antigen receptor type, thus producing a targeted response to a specific antigen (Abbas et al., 2017). After exposure, B cells then transform into plasma cells, produce antibodies, and target microbes for phagocytosis through a process known as humoral immunity (Abbas et al., 2017). Antibodies (also known as immunoglobulins) are the main mechanism through which the adaptive immune system recognizes and targets microbes for elimination (Abbas et al., 2017). Antibodies can be divided into two primary components: the antigen-binding portion, which varies widely to allow for response to a multitude of antigens, and the non-binding portions, which serves more general functions. There are five main isotypes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM; each of which serves a distinct function (Abbas et al., 2017). The most abundant antibody type is IgG, which promotes phagocytosis of microbes by coating of microbes with antibodies that tag them for destruction by mononuclear phagocytes and neutrophils (Abbas et al., 2017). IgA antibodies are primarily responsible for mucosal immunity, or secretion of immunoglobulins into the gastrointestinal and respiratory lumens to defend against oral or airborne pathogens (Abbas et al., 2017). IgM antibodies promote microbe

phagocytosis and lysis, and stimulate inflammation via mast cells, neutrophils, and endothelial cells (Abbas et al., 2017). IgD and IgE are the least prevalent subtypes, and are involved in B cell maturation and eosinophil activation, respectively (Abbas et al., 2017). Following antigen stimulation, a subset of B cells expressing high-affinity antigen receptors remain in the blood and lymphoid organs for long periods of time (Abbas et al., 2017). These so-called memory B cells serve to rapidly respond to future reinfection (Abbas et al., 2017). As antibody response is an essential component of humoral immunity, any deficits in antibody production can lead to increased susceptibility to infection and disease.

Also derived from lymphoid progenitor cells are T cells, which are activated by cytokine release from B cells via a process known as cell-mediated immunity (Abbas et al., 2017). There are two main subpopulations of T lymphocytes that are differentiated by the expression of their surface markers CD4 and CD8: CD4+ helper T cells and CD8+ cytotoxic T lymphocytes (CTLs) (Abbas et al., 2017). CD4+ helper T cells differentiate into effector cells, travel to the site of infection, and secrete cytokines to recruit other cell types or promote phagocytosis by neutrophils and macrophages. CD8+ CTLs destroy microbes directly (Abbas et al., 2017). Together, B and T lymphocyte activation encourages the destruction of infection reservoirs through antibody production and phagocytosis.

Deficits in the immune system can lead to immunosuppression, either primary/congenital or secondary/acquired (Abbas et al., 2017). Immunodeficiencies increase susceptibility to infection through defects in lymphocyte development or innate and/or adaptive immunity (Abbas et al., 2017). Two major types of immunodeficiencies are B/T cell loss and antibody deficiencies, which can increase susceptibility to infection (Abbas et al.,

2017). Acquired immunosuppression often occurs due to drug exposure (Abbas et al., 2017). Opioids are generally considered immunosuppressive, though the strength of this effect is variable and depends on the type of opioid used (Budd, 2006). Opioid-induced immunosuppression occurs either through the direct action of opioids on immune cells, via μ-opioid receptors, or indirectly through the release of corticosterone and other immunosuppressive factors (Budd, 2006). Opioid receptors, including μ-, δ-, and κ-opioid receptors, have been detected both in humans and rodents on B and T lymphocytes, monocytes, macrophages, splenocytes, granulocytes, dendritic cells, and microglia (Machelska and Celik, 2020).

1.3.4 Evidence from adult rodents and humans

Opioid-induced immunosuppression is a growing clinical problem. Recent opioid use, particularly long-acting opioids, has been associated with an increased risk of pneumonia (Dublin et al., 2011). Intravenous drug users frequently present to the emergency room with pneumonia and soft-tissue infections, although lifestyle factors associated with illicit drug use likely complicate this relationship (Palepu et al., 2001).

Chronic morphine use leads to deficits in immune cell function, increased microbial replication, and susceptibility to infection in a μ-opioid receptor-dependent manner. One deficit commonly associated with chronic opioid use is decreased natural killer cell cytotoxicity (NKCC). Natural killer cells (NKs) are a member of the innate immune system and serve as the first line of defense against infection by phagocytosing foreign material and releasing cytokines to activate macrophages (Abbas et al., 2017). Male heroin users, male and female surgical patients receiving opioid anesthesia, and female volunteers treated with morphine (Beilin et al., 1996;

Novick et al., 1989; Yokota et al., 2004) all had decreased NKCC. Similar results have been reported in male rodents (Beilin et al., 1992, 1989; Fecho and Lysle, 1999; Nelson et al., 2000; Sacerdote et al., 1997). This opioid-induced attenuation in NKCC activity is μ-opioid receptor-dependent, as the effect is blocked by pre-treatment with μ-opioid receptor antagonists (Beilin et al., 1989; Nelson et al., 2000). Morphine's effects on NKCC are centrally mediated, as systemic treatment of mice with N-methyl-morphine, which does not cross the blood-brain barrier, does not affect NKCC (Shavit et al., 1986). The midbrain periaqueductal gray (PAG), an essential part of the downstream pain modulation pathway with high μ-opioid receptor expression (Gutstein et al., 1998), has been implicated in morphine's effect. Intra-PAG administration of morphine decreases NKCC in male rats (Liang-Suo et al., 2002; Lysle et al., 1996; Weber and Pert, 1989), an effect blocked by naltrexone pretreatment (Weber and Pert, 1989), suggesting that the action of morphine on μ-opioid receptors in the PAG is necessary and sufficient for opioid-induced suppression of NKCC. Paralleling decreased NKCC is the morphine-induced decrease in macrophage phagocytosis. Morphine- (Casellas et al., 1991; Lugo-Chinchilla et al., 2006; Tomei and Renaud, 1997) or DAMGO-treated (Tomassini et al., 2004) macrophages derived from female mice have decreased phagocytotic activity via a μ-opioid receptor-dependent mechanism (Casellas et al., 1991; Lugo-Chinchilla et al., 2006). Decreased phagocytosis of microbes by natural killer cells and macrophages leads to increased microbial replication, pathogen load, and disease severity, suggesting that opioid exposure increases both the mortality and morbidity of infection.

Morphine also decreases antibody production. Murine splenocytes incubated with morphine (Eisenstein et al., 1993; Taub et al., 1991) or male and female mice implanted with 75

mg morphine pellets (Bussiere et al., 1992) produced fewer antibodies in response to antigen presentation. One possible mechanism for this decreased antibody production is the failure of antigen-presenting cells (APCs) to present antigens to lymphocytes. Indeed, APCs from morphine-treated female mice were less effective at presenting antigens to T cells due to a 23% decrease in major histocompatibility complex II (MHC II) expression on APCs (Eisenstein et al., 1993). Class II MHC molecules bind peptides and help present antigens to CD4+ T cells, which is necessary for their activation and phagocytic activity (Abbas et al., 2017). Decreased antibody production reduces the impact of humoral immunity on infection, leading to prolonged illness and increased pathogen load.

Morphine also decreases leukocyte migration and lymphocyte differentiation due to alterations in cytokine release. Cytokines inhibited by morphine exposure include IFN-α (Stoll-Keller et al., 1997), IL-1β, IL-6, G-CSF, KC (Clark et al., 2007), TNF-α (Clark et al., 2007; Madera-Salcedo et al., 2011) and IL-23 (Wang et al., 2011). Morphine also promotes Th2 differentiation of T lymphocytes due to upregulation of Th2-promoting cytokines (IL-4, IL-5, and IL-10) and downregulation of Th1-promoting cytokines (IL-2, IFN-γ, and T-bet) (Roy et al., 2005, 2004). Th1 lymphocytes are the primary effector cells for phagocytic macrophages and Th2 lymphocytes mediate phagocytosis-independent defense mechanisms; therefore, this bias could increase pathogen load and disease severity after morphine exposure. Morphine's effects on cytokine release are likely due, at least in part, to its suppressive impact on transcription factors, including the inducible transcription factor NF-κB (Wang et al., 2003; Welters et al., 2000). Decreased cytokine production during the innate immune system phase reduces adaptive immune system activation. Furthermore, suppressed cytokine release during the adaptive

immune system phase attenuates lymphocyte activation and phagocytosis, increasing morbidity and mortality due to infection.

There are contradictory opinions on the clinical relevance of opioid-induced immunosuppression (Budd, 2006). However, microbial replication, disease severity, and mortality is increased with long-term administration of morphine to humans and rodents, suggesting that the suppression of immune cell functions observed following opioid exposure has real health implications. Morphine-exposed human cell cultures have increased viral replication of hepatitis C (Li et al., 2003; C. Q. Wang et al., 2005) and HIV-1 (Homan et al., 2002; Steele et al., 2003), and male and female mice exposed to chronic morphine have increased mortality rates to Salmonella pneumoniae (J. Wang et al., 2005), Salmonella enteritidis, Listeria monocytogenes (Asakura et al., 2006), and Salmonella enterica infection (Breslow et al., 2010). Morphine also promotes increased viral and bacterial load in male Rhesus macaques infected with SIVmac239 (Suzuki et al., 2002), male mice infected with Salmonella pneumoniae (J. Wang et al., 2005), female mice inoculated with Salmonella typhimurium (Feng et al., 2006), and rats infected with rat-adapted influenza virus (Coussons-Read et al., 1998). Lastly, mice exposed to morphine and injected with the experimental immune activator lipopolysaccharide (LPS) had increased mortality vs. mice injected with LPS alone (Roy et al., 1999). Together, this evidence suggests that opioids not only suppress peripheral immune function but have severe implications for morbidity and mortality after infection.

1.3.5 Impact of opioids on the central nervous system

The brain is considered an “immuno-privileged” organ, where foreign antigens are generally tolerated (Abbas et al., 2017). The blood-brain barrier prevents the migration of

immune cells into the brain, impairing the initiation of adaptive immunity (Abbas et al., 2017). However, resident macrophages of the brain, termed microglia, respond to tissue damage, infection, or inflammation as part of the innate immune system (Abbas et al., 2017). Although morphine typically leads to peripheral immunosuppression, its effects on microglia generally are stimulatory. Morphine binds to the same binding pocket as LPS in the TLR4/MD-2 complex on microglia and produces neuroinflammation through TLR4 oligomerization, leading to p38 and ERK phosphorylation, NF-κB activation, and ultimately cytokine production (Wang et al., 2012). Morphine also binds to μ-opioid receptors on microglia, promoting proinflammation via activation of PKCε, Akt, and ERK1/2 (Merighi et al., 2013) (see Figure 1-2).

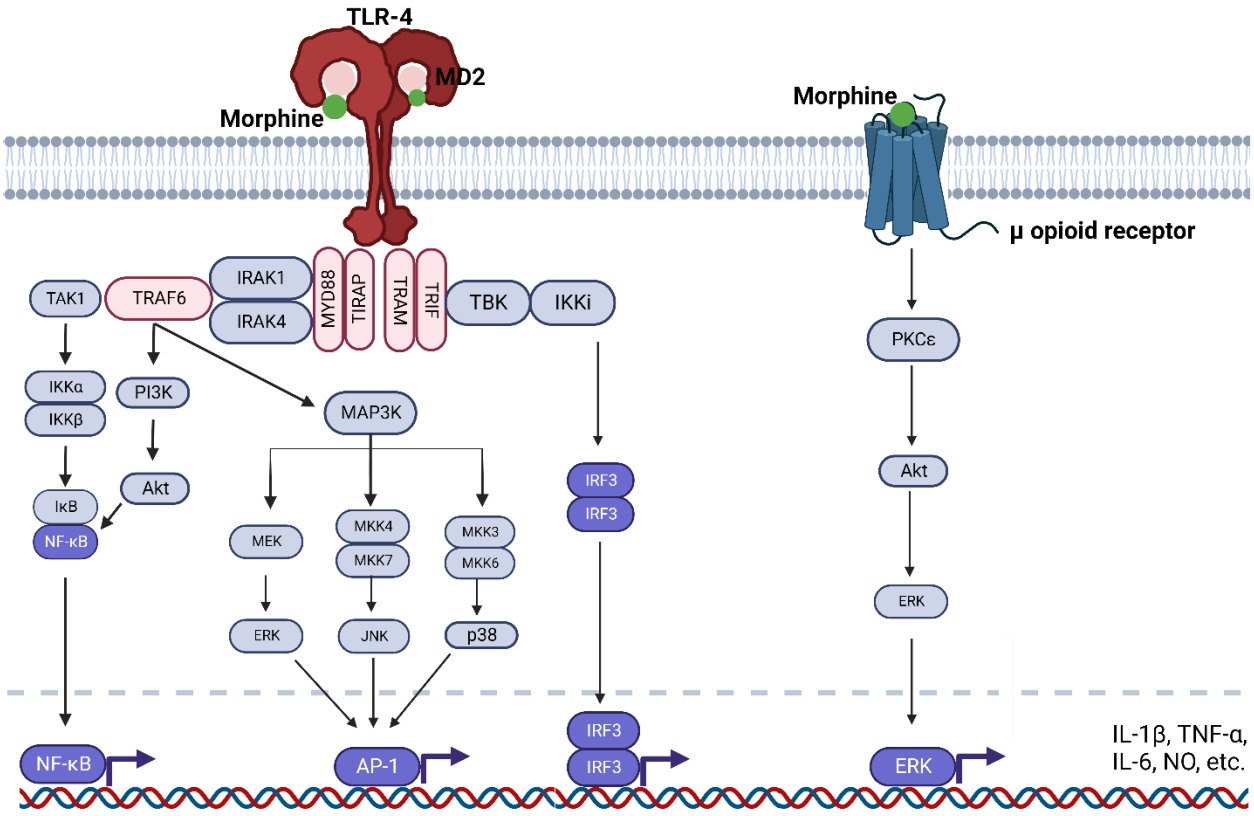


Figure 1-2. Morphine's effects on microglia via TLR4 and μ opioid receptor binding. Created with Biorender.

Male mice implanted with morphine pellets had increased CD11b expression, a measure of microglia activation, and increased TNF-α and IL-6 release from microglia (Bokhari et al., 2009; Zhang et al., 2011). Intermittent morphine exposure in male mice also increases microglial soma size, another measure of activation (Lee et al., 2018). Functionally, morphine promotes phagocytosis in primary microglial human cultures (Lipovsky et al., 1998; Peterson et al., 1995). Together, this evidence suggests that microglia are activated by morphine exposure, an effect opposite to what is observed in the peripheral immune system (see Figure 1-3).

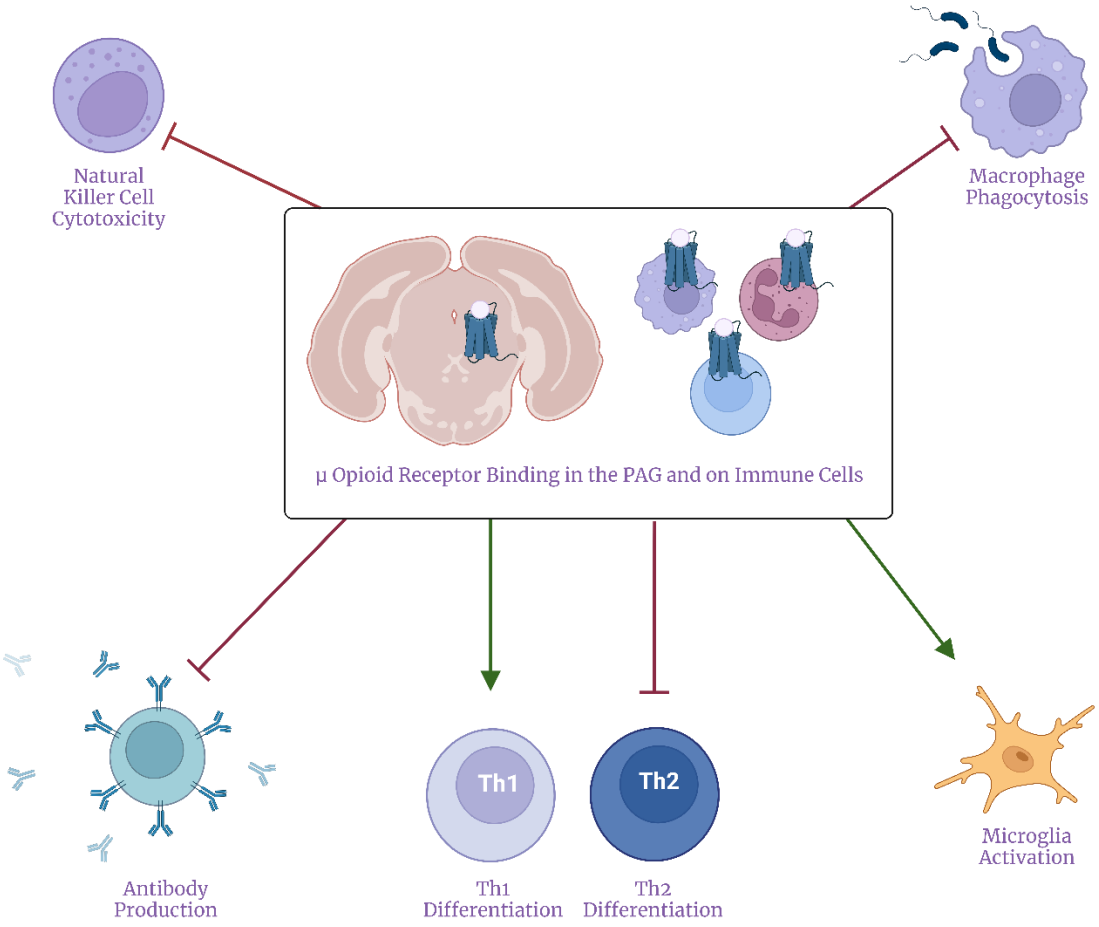


Figure 1-3. Effects of morphine on immune functioning in adult humans and rodents. Created with Biorender.

1.3.6 Evidence from perinatal opioid exposure in rodents and humans

Limited evidence exists regarding the long-term immune consequences of perinatal opioid exposure in rodents. Male and female rats perinatally exposed to the long-acting opioid l-alpha-acetylmethadol (LAAM) or male rats exposed to morphine from E12-18 displayed a blunted fever in response to LPS treatment (Hamilton et al., 2007; Shavit et al., 1998). Morphine exposure from E12-18 in male rats also suppressed natural killer cell cytotoxicity (Shavit et al., 1998). The mechanisms underlying perinatal opioid-induced immunosuppression are not fully elucidated; however, one potential contribution includes a reduction in immune cell number and function. Indeed, blood from children exposed to opioids *in utero* had a reduction in neutrophil levels resulting in lower neutrophil-to-lymphocyte ratio, decreased concentrations of IL-10, IL-12p70, IL-1β, IL-2, IL-4, and IL-6 (Miller et al., 2022), and an elevated risk of lymphopenia (decreased number of lymphocytes) (Adatia et al., 2021; Romanos-Sirakis et al., 2020). Retrospective chart reviews have associated NOWS with an increased risk of hospitalization during early life (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017). Particularly relevant is an increased adjusted relative risk for infectious and parasitic diseases, which was maintained even after adjustment for gestational age and other confounding factors (Witt et al., 2017). Together, these studies suggest that perinatal opioid exposure in rodents and humans leads to long-term deficits in immune functioning, likely via decreases in the number and function of lymphocytes, thereby increasing the risk for infection across the lifespan.

1.3.7 Overview of the gut microbiota and its effect on immune system development

The gut microbiota, or the collection of commensal bacteria living in the intestinal mucosal surface or the gut lumen, consists of approximately 1014 microbes and 600,000 genes (Abbas et al., 2017; World Gastroenterology Organization, 2014). These bacteria generally do not elicit immune responses (Abbas et al., 2017), but rather help to train and develop the immune system's natural functions. Studies utilizing germ-free mice, which are sterile and thus do not contain gut microbiota, suggest that commensal microbes during early development are necessary for proper immune system maturation (World Gastroenterology Organization, 2014). Repeated exposure to commensal bacteria across development trains the immune system to develop tolerance in order to prevent autoimmunity (Abbas et al., 2017; World Gastroenterology Organization, 2014).

The gastrointestinal (GI) immune system is the largest subsystem of the immune system, both in the number of lymphocytes and in antibody production (Abbas et al., 2017). The gastrointestinal tract consists of three major parts: the intestinal lumen, where commensal bacteria and potential pathogens reside; the lamina propria, where immune cells survey the GI tract; and the mucosal epithelial layer, which forms a barrier between the two (Abbas et al., 2017) (see Figure 1-4). Tight junction proteins connect epithelial cells in the mucosal epithelial layer to block microbial transport between cells (Abbas et al., 2017). Epithelial cell types include goblet cells, which produce a thick mucus to further separate the lamina propria and commensal bacteria in the gut lumen; M cells, which sample antigens and directly transport those antigens to naïve lymphocytes; and Paneth cells, which secrete antibacterial peptides to kill bacteria that have transversed the mucosal layer (Abbas et al., 2017). Overall, the GI tract is

designed to sample the intestinal lumen contents and minimize immune responses provoked by commensal bacteria of the gut microbiota.

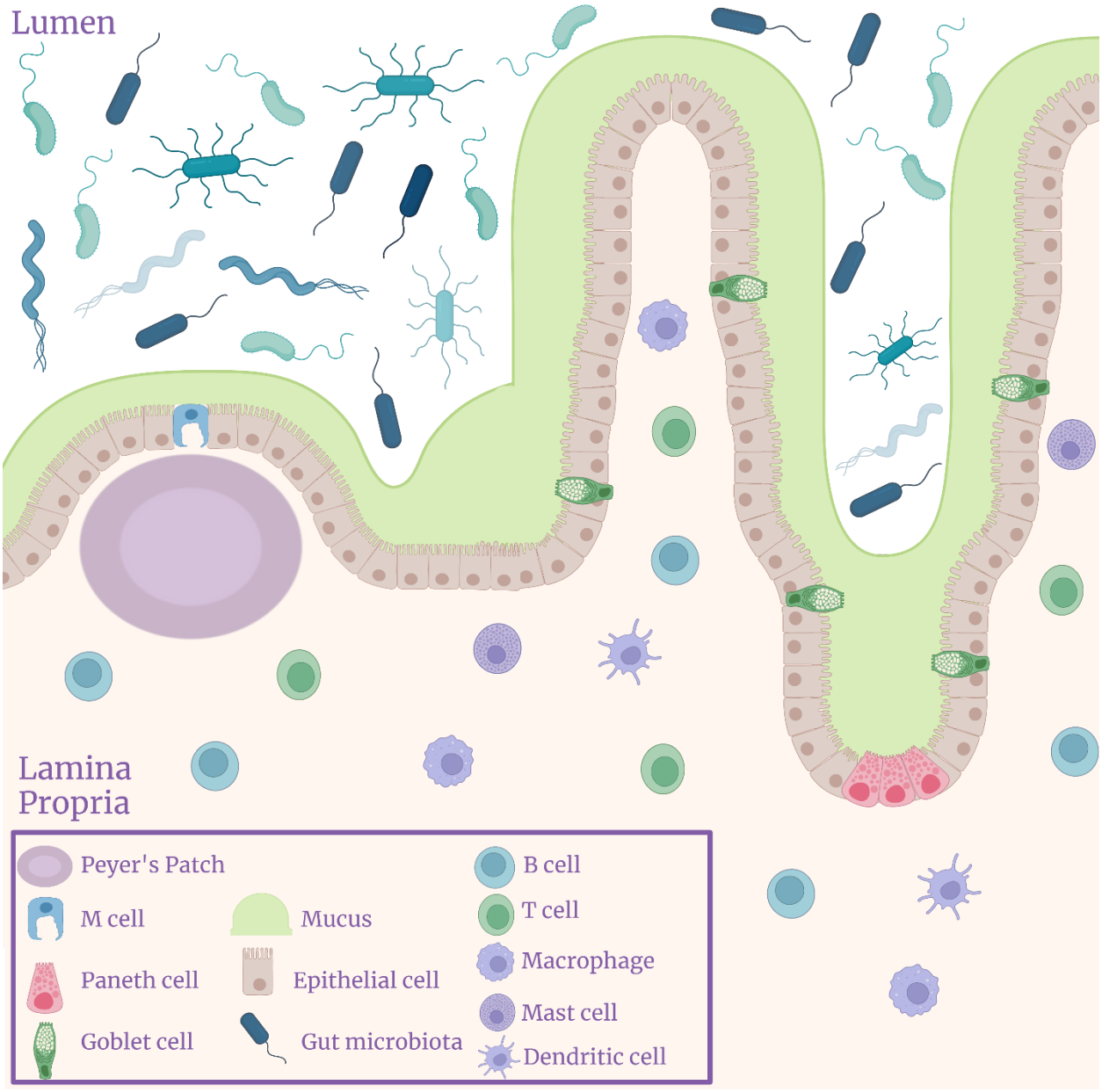


Figure 1-4. Overview of the gut lumen and lamina propria. Created with Biorender.

Innate immunity in the gut largely relies on the integrity of the epithelial cell layer so that bacterial contact with immune cells in the lamina propria is minimized. The epithelial cell

layer has several features to ensure this separation, including a thick mucus layer, natural antibiotic molecules known as defensins, and tight junction proteins to minimize paracellular transport. The epithelial layer also expresses pattern recognition receptors, including TLRs and NOD-like receptors (NLRs) (Abbas et al., 2017), which primarily serve to respond to foreign antigens such as bacteria and initiate the innate immune system cascade. During development, repeated exposure to commensal bacteria promotes the development of tolerance to these microbes to prevent autoimmunity, but concurrently promoting inflammation following contact with potentially pathogenic bacteria (Abbas et al., 2017). Dysbiosis, or altered gut microbiota composition, can influence immune system tolerance by inducing inflammation in response to commensal bacteria, potentially leading to autoimmunity.

Adaptive immunity in the gut is primarily mediated by B-cell-produced, IgA-mediated humoral immunity (Abbas et al., 2017). T cell subtypes in the gut are predominantly helper T cells and regulatory T cells, which help to promote and maintain tolerance against commensal bacteria (Abbas et al., 2017). Microfold (M) cells in the epithelial cell layer directly deliver antigens to dendritic cells and naïve B cells located in Peyer's patches, where lymphocytes differentiate into effector cells (Abbas et al., 2017). These lymphocytes are produced mainly in mesenteric lymph nodes, and are then recruited to the lamina propria via integrin expression (Abbas et al., 2017). Effector cells then produce large amounts of IgA antibodies, which are transported back into the lumen by poly-Ig receptors (Abbas et al., 2017). IgA production primarily occurs in response to a loss of gut barrier integrity, meaning the immune system is contacted by commensal bacteria, and promotes agglutination, mucus production, or bacterial clearance (Mantis et al., 2011), making IgA an essential aspect of adaptive immunity in the gut.

The gut microbiota is necessary for the proper development and maturity of the gastrointestinal immune system. Gut epithelial barrier repair after injury or infection, an essential portion of innate immunity, requires stimulation from commensal bacteria (Abbas et al., 2017). Similarly, B cell class switching to produce IgA, a major portion of adaptive immunity, requires bacterial exposure (Abbas et al., 2017). Alteration in gut microbiota composition impacts the proper development of the gastrointestinal immune system, as repeated exposure to commensal bacteria during development promotes tolerance and minimizes autoimmunity and allergy (Abbas et al., 2017). These findings have led to the formation of the "hygiene hypothesis," wherein exposure to bacteria in early postnatal life helps to regulate and adequately mature the immune system (Abbas et al., 2017). Dysbiosis alters immune system maturation during a crucial developmental period, leading to long-term issues with tolerance and autoimmunity.

The gut microbiota is acquired at delivery via the vaginal microbiota (or skin microbiota in the case of Caesarean sections) (World Gastroenterology Organization, 2014). In humans, the gut microbiota is highly variable during early life and does not reach an adult-like state until three years of age (World Gastroenterology Organization, 2014). The composition of the maternal microbiota is the primary driver of offspring gut microbiota composition (Nyangahu et al., 2018). Furthermore, breastfeeding introduces bacteria, prebiotic fibers, and IgA antibodies into the gut to shape the gut microbiota's composition (Mulligan and Friedman, 2017), leading to a prolonged impact on the gut microbiota of the offspring. The early postnatal period is a crucial window for gut microbiota development, and any perturbations (drug exposure, poor diet, disease, etc.) can produce long-term changes in immune system function.

The GI tract also includes hundreds of millions of neurons, entitled the enteric nervous system (ENS), located in the ganglia of the myenteric and submucosal plexus (Holzer et al., 2001). Major neurotransmitters of the ENS include acetylcholine, tachykinins, serotonin, and, most importantly, endogenous opioids (Holzer et al., 2001). μ-opioid receptors are highly expressed in the ENS, particularly in the proximal colon (Fickel et al., 1997). The ENS primarily promotes peristalsis, or propulsion of luminal contents down the gut (Holzer et al., 2001). Opioids can therefore have indirect effects on gut health via the microbiota and immune system, as well as acting directly on the neurons in the gastrointestinal system.

1.3.8 Impact of morphine on the gut microbiota in adult humans and rodents

Activation of opioid receptors in the ENS inhibits peristalsis and slows intestinal transit, producing constipation, bacterial overgrowth, and gut microbiota dysbiosis (Holzer et al., 2001). Treatment of human volunteers with a low dose of morphine significantly increased gastric emptying half-times and small intestinal transit times (Yukioka et al., 1987). Similarly, 75 mg morphine pellet implantation in female mice led to a 38% reduction in intestinal transit (Feng et al., 2006). As slowed intestinal transit has been associated with bacterial overgrowth in the small intestine (Roland et al., 2015), inhibition of peristalsis may be a causative factor in morphine-induced gut microbiota dysbiosis.

Morphine also increases gut epithelial barrier permeability, allowing for bacterial dissemination into the lamina propria and beyond. Implantation of 25 or 75 mg morphine pellets into adult female mice led to increased bacterial dissemination to the mesenteric lymph nodes (MLNs) and liver due to disruptions in tight junction organization (Meng et al., 2013; Wang et al., 2020). This effect was mediated by morphine acting on μ-opioid receptors, as no

alteration in tight junction organization occurred in μ-opioid receptor knockout (MORKO) mice (Meng et al., 2013). Morphine treatment in male and female mice also led to Gram-negative bacteremia (presence of bacteria in the blood) and bacterial dissemination to the liver, spleen, kidneys, heart, and lungs; this effect was blocked by naloxone co-treatment (Bhaskaran et al., 2001). Increased bacterial dissemination into the blood and organs from the gut can lead to sepsis and death; indeed, prescription opioids have been associated with increased mortality from sepsis (Zhang et al., 2018).

Morphine treatment in adult humans and rodents is known to induce changes in alpha diversity, or the number and evenness of microbial taxa present in a sample, and beta diversity, or the similarity/dissimilarity between groups. Treatment of male mice with intermittent morphine alters beta diversity vs. saline controls (Lee et al., 2018) and, in particular, increased Ruminococcus and decreased Lactobacillus (Lee et al., 2018). Ruminococcus species have been associated with Crohn's disease and other inflammatory conditions (Henke et al., 2019) and Lactobacillus species are generally considered probiotic and anti-inflammatory (Reid, 1999). This suggests that the morphine-exposed gut is generally proinflammatory and susceptible to disease. Other studies have identified increases in potential pathogens after morphine treatment, including *Flavobacterium, Enterococcus, Fusobacterium, Sutterella*, and *Clostridium*, along with reductions in alpha diversity (Wang et al., 2018). Human studies have also associated opioid use with decreased alpha diversity and expansion of the potential pathogen *Clostridium* (Gicquelais et al., 2020). Reduced alpha diversity has been associated with inflammatory bowel disease (IBD) and systemic inflammation (Lozupone et al., 2012), suggesting that opioid exposure promotes proinflammatory changes in diversity associated with gastrointestinal

disease states. Morphine-treated mice also had a reduction in the ratio of the phyla Bacteroidetes and Firmicutes, which has also been associated with obesity and aging (Banerjee et al., 2016; Meng et al., 2013). Co-administration of naloxone, as well as morphine treatment in TLR2 knockout or MORKO mice, prevents morphine's effects on gut microbiota composition (Banerjee et al., 2016). This evidence suggests that morphine exposure in adult rodents promotes a proinflammatory, less diverse, and potentially pathogenic microbiota composition.

Functionally, morphine can lead to decreased bile acid production (end products of cholesterol metabolism) that is associated with gut barrier disruption and inflammation (Banerjee et al., 2016; Wang et al., 2018). This reduction in bile acid production is likely due to decreased abundance in operational taxonomic units (OTUs), including Lactobacillus, which deconjugate bile acids (Banerjee et al., 2016). Reduced bile acid production is also associated with increased cholesterol and coprostanol levels in morphine-treated mice, as these molecules are precursors that would normally be metabolized into bile acids in healthy rodents (Banerjee et al., 2016). Similarly, the production of short-chain fatty acids (SCFAs), involved in energy homeostasis, gut barrier function, and intestinal immunity, is also reduced, which can exacerbate morphine-induced gut barrier permeability and inflammation, promoting bacterial dissemination and sepsis.

Morphine-induced gut microbiota composition alterations also produce neuroinflammation via microglial activation. Transplantation of the gut microbiota from morphine-exposed mice to antibiotic-depleted mice, which have had their microbiota knocked down, led to increased microglial cell body size, indicative of activation (Lee et al., 2018). The gut microbiota communicates to the CNS via the vagus nerve and/or the release of neuroactive

metabolites into circulation, including SCFAs (Abdel-Haq et al., 2019). Gut microbiota signaling to the brain can promote and/or exacerbate morphine-induced microglial activation, leading to increased neuroinflammation and microglial cytokine release, potentially via alterations in SCFA production.

Alterations in gut microbiota composition following morphine treatment increase susceptibility to pathogenic bacteria, leading to possible sepsis and death. Morphine-treated mice infected with Citrobacter rodentium had increased bacterial dissemination due to decreased gut barrier integrity, which increased bacterial load (Wang et al., 2020). Morphine also reduced the number of mucus-secreting goblet cells, potentially decreasing the thickness of the mucus layer and allowing bacteria to more easily approach, and potentially cross, the epithelial cell layer to reach immune cells in the lamina propria (Wang et al., 2020). Similarly, morphine-treated mice subjected to cecal ligation and puncture to induce sepsis had increased bacterial translocation, bacterial load, and decreased bacterial clearance (Meng et al., 2015). Morphine is well known to promote the progression of sepsis and increase susceptibility to septic shock (Hilburger et al., 1997; Ocasio et al., 2004; Roy et al., 1999), suggesting that morphine-induced decreases in gut barrier permeability and microbiota composition have tangible consequences for the health of humans and rodents alike.

1.3.9 Impact of perinatal morphine on gut microbiota development

One of the hallmark symptoms of NOWS is gastrointestinal distress, including diarrhea and cramping, which often manifests as poor feeding and irritability (Maguire and Gröer, 2016). The effects of morphine in adult humans and rodents have led to a theoretical model of gastrointestinal distress in offspring exposed to perinatal opioids, wherein opioids bind to fetal

gut μ-opioid receptors in the enteric nervous system, leading to decreased gut motility and decreased peristalsis (Maguire and Gröer, 2016). This, along with the inheritance of dysbiotic gut microbiota from the mother, likely alters gut microbiota composition in offspring exposed to opioids perinatally (Maguire and Gröer, 2016). Synergistic effects of decreased gut motility, dysbiotic gut microbiota, increased gut barrier permeability, and reduced innate and adaptive immune system function during a critical period of development likely have a negative impact on immune system development and training, leading to long-term immunosuppression and increased disease susceptibility.

Preclinical studies of perinatal opioid exposure have shown variable and complex differences in gut microbiota composition. Offspring from female mice that received daily oxycodone two weeks before breeding to parturition showed no overt differences in alpha or beta diversity vs. control mice; however, the relative abundance of specific families and genera was altered in the offspring (Lyu et al., 2022). Oxycodone-exposed male mice had an increased relative abundance of the potentially pathogenic families Coriobacteriaceae and Clostridia, along with decreases in Butyricicoccus and Lactobacillales, which are involved in the production of the anti-inflammatory SCFA butyrate and inositol stereoisomers, respectively (Lyu et al., 2022). Oxycodone-exposed females also had increases in the potential pathogenic family Clostridia and genera Butyricimonas, previously associated with Parkinson's and autism spectrum disorder (Lyu et al., 2022). Increased alpha diversity was also observed in offspring exposed to oxycodone and methadone throughout pregnancy until weaning (Grecco et al., 2021), while beta diversity was significantly altered in the dams but not the offspring, suggesting many complex factors involved in composition of the gut microbiota (Grecco et al.,

2021). Male and female offspring exposed to oxycodone and methadone had an increased abundance of the SCFA-producing Lachnospiraceae NK4A136, which was proposed as a potential signature of opioid-exposed microbiota (Grecco et al., 2021).

Very few clinical studies concerning NOWS and gut microbiota composition exist currently. NOWS-associated diarrhea has been associated with lower alpha and beta diversity, notably a higher abundance of the phyla Firmicutes, Proteobacteria, and Verrucomicrobia, and a lower abundance of Actinobacteria and Bacteroidetes (Sealschott et al., 2020). Although the evidence is currently mixed on the impact of perinatal opioid exposure on alpha and beta diversity, general findings support an increase in potentially pathogenic bacteria and alterations in bile acid and SCFA production.

1.3.10 Sex differences in immunity and NOWS

Sex has a significant impact on immunity. Women have higher rates of autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, Hashimoto's disease, and Graves' disease (Marriott and Huet-Hudson, 2006). However, females generally have greater adaptive immune responses to antigen challenges and decreased susceptibility to pathogens (Marriott and Huet-Hudson, 2006). Cohort studies in humans report that men have an increased risk of infections after trauma, sepsis susceptibility, and mortality (Marriott and Huet-Hudson, 2006). Endotoxin challenge studies in rats also show sex differences in mortality, with an increased risk of male death (Marriott and Huet-Hudson, 2006). These effects are likely mediated through decreased proinflammatory and increased anti-inflammatory cytokine production in females (Marriott and Huet-Hudson, 2006). Estrogen and non-classical androgen receptors are expressed on most leukocyte subtypes (Lasrado et al., 2020); androgens are

generally considered immunosuppressive and estrogens augment immune responses, potentially leading to autoimmunity (Marriott and Huet-Hudson, 2006). Some sex differences in autoimmune diseases may be due to known differences in gut microbiota composition (Kim et al., 2020; Lasrado et al., 2020). Although specific findings vary due to confounding factors such as weight/diet and drug use, there seems to be an impact of sex hormones on gut microbiota composition, as male and female prepubescent children did not have differential beta diversity (Kim et al., 2020). Male fetuses are traditionally considered more susceptible to prenatal adversity, potentially due to slower development, maternal immunoreactivity, or prenatal hormone exposure (DiPietro and Voegtline, 2017). Together, these studies suggest that perinatal opioid exposure may affect male and female offspring differently, particularly with respect to immune function. However, clinical evidence is mixed on the impact of sex on NOWS risk and severity, with some studies suggesting male infants have a higher risk of NOWS and need for pharmacological treatment without changes in severity (Charles et al., 2017; O'Connor et al., 2013), and others reporting no differences in any measure of NOWS risk or severity (Holbrook and Kaltenbach, 2010; Unger et al., 2011). Preclinical studies rarely include both male and female offspring; those that do seldom compare the sexes and often report contradictory evidence.

1.4 Dissertation aims

Previous clinical and preclinical research investigating the impact of opioids on immune function and gut microbiota composition in adults report that opioids generally promote peripheral immunosuppression, central neuroinflammation, and gut microbiota dysbiosis. Limited research has been conducted on the effects of perinatal opioid exposure on immune

function or gut microbiota composition, but general conclusions support immune dysregulation and gut microbiota dysbiosis in offspring of opioid-treated dams. However, these studies have typically used models of perinatal opioid exposure that do not represent the clinical features of maternal opioid use disorder (MOUD).

Perinatal opioid exposure animal models that intend to mimic MOUD and NOWS should include the following factors: 1) opioid exposure before, during, and post-pregnancy; 2) minimal stress from repeated handling and/or injections; 3) pulsatile drug delivery; and 4) increasing dosing throughout pregnancy to account for tolerance. Most studies investigating perinatal opioid exposure utilize dosing with repeated injections from embryonic day 11-18 (E11-18) or implantation of subcutaneous morphine pellets to produce steady-state opioid levels with added confounds of stress from repeated handling and injections (Harder and Murphy, 2019). Therefore, these studies do not recapitulate the clinical profile of MOUD, and findings using these models should be interpreted with caution. Use of a novel, clinically-relevant model of MOUD and NOWS will enable identification of potential immune deficits resulting from perinatal opioid exposure. In our model, female Sprague Dawley rats are implanted with iPrecio® SMP-200 microinfusion minipumps at P60. Initially, rats are treated with 10 mg/kg morphine three times a day, with doses increasing weekly by 2 mg/kg until 16 mg/kg is reached. Microinfusion pumps are refilled via a subcutaneous injection, thereby negating the need for anesthesia. One week after morphine initiation, females are paired with sexually-experienced males for two weeks to induce pregnancy. Morphine exposure to the dams continues throughout gestation. Just prior to parturition (E18), dosage is decreased to twice-a-day, as initial pilot studies using three-times-a-day delivery led to increased pup

mortality. Dams continue to receive morphine after parturition, so pups receive morphine indirectly through maternal milk. Beginning at P5, the morphine dose is decreased by 2 mg/kg daily until P7, when the dose reaches 0 mg/kg. This protocol mirrors the clinical profile of MOUD and NOWS in that it includes opioid exposure before, during, and after pregnancy, minimizes stress from repeated injections during pregnancy, mimics the peaks and troughs of human drug use, and increases dosing as tolerance and body weight increases. This allows us to better investigate the potential impacts of perinatal opioid exposure on immunity in a clinically translatable manner.

Studies outlined in this dissertation test the hypothesis that morphine exposure alters maternal microbiota composition inherited by the offspring. The dysbiotic gut microbiota then influences immune system development and maturation and synergizes with the direct effects of morphine on the offspring's immune system and gut. Thus, we propose our overarching hypothesis that perinatal opioid exposure leads to altered immune activity in adulthood. Specifically, we hypothesize that male and female rats perinatally exposed to morphine have altered fever response and sickness behavior to LPS, and microglial activation in the ventrolateral periaqueductal gray (PAG), a primary CNS region involved in opioid-induced immunosuppression (Liang-Suo et al., 2002; Lysle et al., 1996; Weber and Pert, 1989). In addition, we hypothesize that these differences are related to altered gut microbiota composition due to previous evidence associating opioid exposure with gut microbiota dysbiosis, including expansion of proinflammatory and potentially pathogenic bacteria. We will test these hypotheses with the following specific aims:

Specific Aim 1: Identify immune deficits in male and female rats perinatally exposed to morphine. In adults, chronic morphine is associated with deficits in natural killer cytotoxicity, macrophage phagocytosis, antibody and cytokine production, lymphocyte proliferation, along with gut microbiota dysbiosis. To date, limited evidence utilizing perinatal exposure to opioids also supports deficits in immune functioning (Hamilton et al., 2007; Shavit et al., 1998) and altered gut microbiota composition (Grecco et al., 2021; Lyu et al., 2022). However, as these studies generally inject dams daily or multiple times a day, often not across all of pregnancy, which is highly stressful and raises concerns about the interpretability of those results. This aim will utilize a clinically-relevant model of perinatal opioid exposure to 1) examine the impact on peripheral immune functioning using behavioral, molecular, and immunological techniques at baseline and following an immune challenge. Furthermore, 2) we will compare microglial activation in the PAG of adult male and female rats perinatally exposed to morphine to saline controls.

Specific Aim 2: Characterize the gut microbiota of male and female rats perinatally exposed to morphine. Although there is ample evidence that chronic opioid exposure in adults alters gut microbiota composition, studies using perinatal opioid exposure have reported complex and variable effects of opioid exposure on gut microbiota composition (Grecco et al., 2021; Lyu et al., 2022). This aim will 1) examine alterations in gut microbiota composition after perinatal morphine exposure, and 2) identify potential therapeutic targets for interventions to ameliorate morphine-induced gut microbiota dysbiosis.

These studies are the first to utilize a clinically relevant model of MOUD and perinatal opioid exposure to describe the role of morphine-induced immunosuppression on adulthood

immune function. Our results identify potential biomarkers and therapeutic targets in the opioid-exposed gut microbiota, and illustrate the necessity for clinically relevant animal models of drug use and abuse.

2 INCREASED LPS-INDUCED FEVER AND SICKNESS BEHAVIOR IN ADULT MALE AND FEMALE RATS PERINATALLY EXPOSED TO MORPHINE

2.1 Introduction

The exponential increase in opioid use in the United States, particularly among women of reproductive age, has resulted in a surge of infants exposed to opioids *in utero*. Most of these infants will experience opioid withdrawal at birth (neonatal opioid withdrawal syndrome; NOWS), requiring an extended stay in the neonatal intensive care unit (Kocherlakota, 2014). Gestation and the early postnatal period are critical periods of immune system development in humans and rodents (Georgountzou and Papadopoulos, 2017); however, little is known about the long-term consequences of perinatal opioid exposure on immune function. In adults, chronic opioid use is associated with suppression of the peripheral immune system, including decreased natural killer cell cytotoxicity (Beilin et al., 1996, 1992, 1989; Fecho and Lysle, 1999; Nelson et al., 2000; Novick et al., 1989; Sacerdote et al., 1997; Yokota et al., 2004), reduced macrophage phagocytosis (Casellas et al., 1991; Lugo-Chinchilla et al., 2006; Tomassini et al., 2004; Tomei and Renaud, 1997), and altered proinflammatory cytokine production (Clark et al., 2007; Madera-Salcedo et al., 2011; Stoll-Keller et al., 1997; Wang et al., 2011). Clinical chart review suggests that infants exposed to opioids *in utero* are similarly at an increased risk of infection and rehospitalization (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017), suggesting parallel suppression of immune function. Chronic opioid-induced deficits in antibody production are of particular concern to opioid-exposed infants (Bussiere et al., 1992; Eisenstein et al., 1993; Taub et al., 1991), as this response is an essential component of the adaptive immune system and serves to form immunological memories of previous exposure. Indeed,

deficits in antibody production increase susceptibility to infection, as the adaptive immune system is unable to recognize and eliminate pathogens (Barmettler et al., 2018).

The periaqueductal gray (PAG), a critical neural substrate in opioid signaling (Loyd et al., 2008), has been implicated in the central immunosuppressive effects of opioids (Gomez-Flores and Weber, 2000). Direct administration of morphine into the PAG suppresses a number of immune cell functions, including natural killer cell cytotoxic activity, lymphocyte proliferation, and cytokine production (Gomez-Flores et al., 1999; Weber and Pert, 1989). Furthermore, morphine has a stimulatory effect on PAG microglia to induce cytokine release in a Toll-like receptor 4 (TLR4)-dependent manner (Bokhari et al., 2009; Eidson and Murphy, 2013; Lee et al., 2018; Zhang et al., 2020, 2011). Morphine has also been shown to suppress peripheral immune cell activity in a TLR4-dependent manner (Zhang et al., 2020). Therefore, morphine is poised to impact the immune system at multiple levels, promoting neuroinflammation centrally, and immunosuppression and increased susceptibility to pathogens peripherally.

Systemic administration of lipopolysaccharide (LPS), which mimics a Gram-negative bacterial infection, is one of the most common experimental immune models (Lasselin et al., 2020). LPS is a pathogen-associated molecular pattern (PAMP) that binds primarily to the innate immune receptor TLR4, leading to peripheral cytokine production (Zampronio et al., 2015). These cytokines act primarily within the hypothalamic median preoptic area to induce prostaglandin E2 synthesis, promoting fever via increased brown adipose tissue metabolism and vasoconstriction (Hart, 1988; Machado et al., 2020; Saper and Breder, 1994; Zampronio et al., 2015). LPS-induced fever is accompanied by a characteristic set of behaviors associated with sickness, including anorexia, lethargy, and reduced grooming (Hart, 1988). Both fever and

sickness serve to engage the immune system to restrict pathogen growth and reduce metabolic demand to facilitate fever (Hart, 1988); therefore, any alterations in the course of a typical fever and sickness response may increase the severity of infection. Counterintuitively, immunosuppression generally leads to elevated febrile response to LPS (Miñano et al., 2004; Tavares et al., 2006, 2005) or infection (Oude Nijhuis et al., 2002) and is often the only sign of infection in immunosuppressed patients (Pizzo, 1999). Although the mechanism by which immunosuppression augments infection-induced fevers is currently unknown, it is thought that immunosuppression leads to an inability of the body to properly mount a cytokine response to infection, leading to an elevated, centrally-mediated fever response (Oude Nijhuis et al., 2002).

Limited preclinical evidence has associated perinatal opioid exposure with immune dysregulation after LPS exposure (Hamilton et al., 2007; Shavit et al., 1998). Previous studies examining the impact of perinatal opioid exposure on immune function utilized dosing paradigms that fail to mirror the clinical profile of women who use opioids while pregnant. The present studies were conducted to address this gap using a clinically relevant and translatable model of perinatal opioid exposure (POE). We hypothesize that perinatal exposure to opioids results in immune system dysregulation, both centrally and peripherally, leading to an increased immune response to LPS.

2.2 Methods

2.2.1 Experimental subjects

All experiments utilized male and female Sprague Dawley rats (Charles River Laboratories, Boston, MA). Rats were housed in same-sex pairs or groups of three on a 12:12 hours light/dark cycle (lights on at 8:00 AM) in Optirat GenII individually ventilated cages

(Animal Care Systems, Centennial, Colorado, USA) with corncob bedding. Food (Lab Diet 5001 or Lab Diet 5015 for breeding pairs, St. Louis, MO, USA) and water were provided ad libitum throughout the experiment, except during testing. These studies were approved by the Institutional Animal Care and Use Committee at Georgia State University and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to reduce the number of rats used and minimize pain and suffering.

2.2.2 Perinatal opioid exposure paradigm

Female Sprague Dawley rats (P60) were implanted with iPrecio® SMP-200 microinfusion minipumps under isoflurane anesthesia (Harder et al., 2023). See Figure 2-1 for a description of the dosing paradigm. Pumps were programmed to deliver morphine at 10 mg/kg three times a day. One week after morphine initiation, females were paired with sexually-experienced males for two weeks to induce pregnancy. Morphine exposure to the dams continued throughout gestation, with doses increasing weekly by 2 mg/kg until 16 mg/kg was reached. Dams continued to receive morphine after parturition, such that pups received morphine indirectly. Beginning at P5, morphine dosage was decreased by 2 mg/kg daily until P7, when the dose reached 0 mg/kg. Control rats were implanted with pumps filled with sterile saline. No differences were noted in maternal behavior of morphine vs. vehicle dams (Harder et al., 2023). Pups were weaned at P21 into treatment-matched cages, where they remained until adulthood.

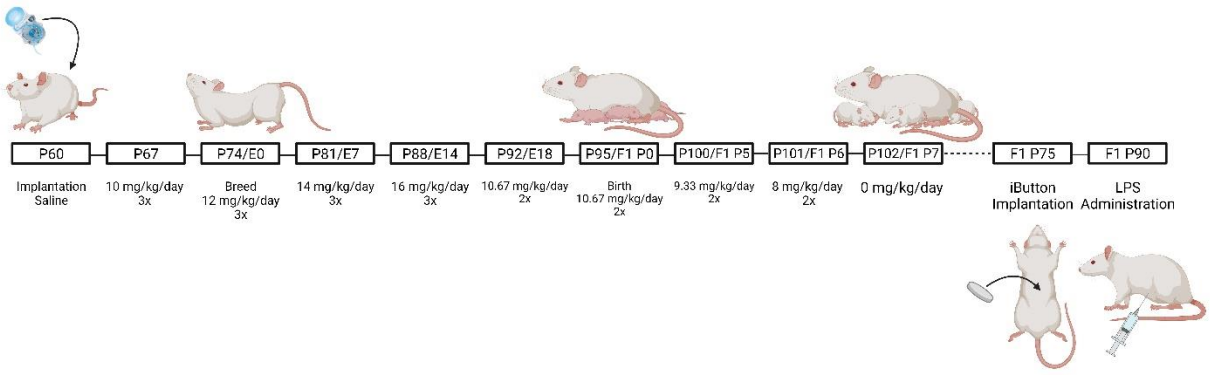


Figure 2-1. Schematic of the perinatal opioid exposure dosing paradigm. Created with Biorender.

2.2.3 iButton implantation

At P75, male and female rats born to mothers exposed to morphine (MOR) or vehicle (VEH) were anesthetized with 5% isoflurane and maintained at 2-3%. A midline abdominal incision was made using sterile surgical techniques, and a wax-coated iButton temperature logger (Thermochron DS1922L) was placed into the abdominal cavity. All rats received carprofen (5 mg/mL/kg; i.p.) prior to and twenty-four hours post-surgery for pain relief. iButton loggers were programmed to record core body temperature in 10-minute intervals beginning twenty-four hours before and twenty-four post-LPS administration.

2.2.4 Lipopolysaccharide treatment

Approximately 14 days following iButton implantation, rats were administered lipopolysaccharide (250 ug/kg/mL, i.p.) derived from E. coli O111:B4 (Sigma-Aldrich; L2630) to induce fever and sickness. Body temperature was recorded for 24 hours post-LPS. Every two hours, LPS-induced sickness behavior was quantified using an 8-point Likert scale, which measured changes in physical features (ear changes, nose/cheek flattening, orbital tightening,

and piloerection), modified from (Sotocinal et al., 2011). See Figure 2-2 for an example of sickness-associated features. Rats received a score of 0 if absent, 1 if present, and 2 if present and severe, for a maximum sickness score of 8.

Figure 2-2. Example of sickness-associated features (ears laid back against the head, nose/cheek flattened, squinted eyes, and piloerect fur). Created with Biorender.

2.2.5 Cytokine array

Analysis of 24-hour data identified 8 hours post-LPS as the time point when the largest differences in fever and sickness behavior were observed; therefore, a separate group of rats was decapitated at eight hours post-LPS. Terminal blood was collected in EDTA tubes and centrifuged (4°C, 3000g, 15 minutes) to collect plasma. A commercially available, slide-based cytokine microarray (Abcam ab197484) was used to quantify levels of the following cytokines: IFN-γ, IL-1α, IL-1β, and IL-10. Slides were visualized using a Cy3 laser scanner (GenePix® 4000B), and median signal values from four replicates per cytokine were compared to standards.

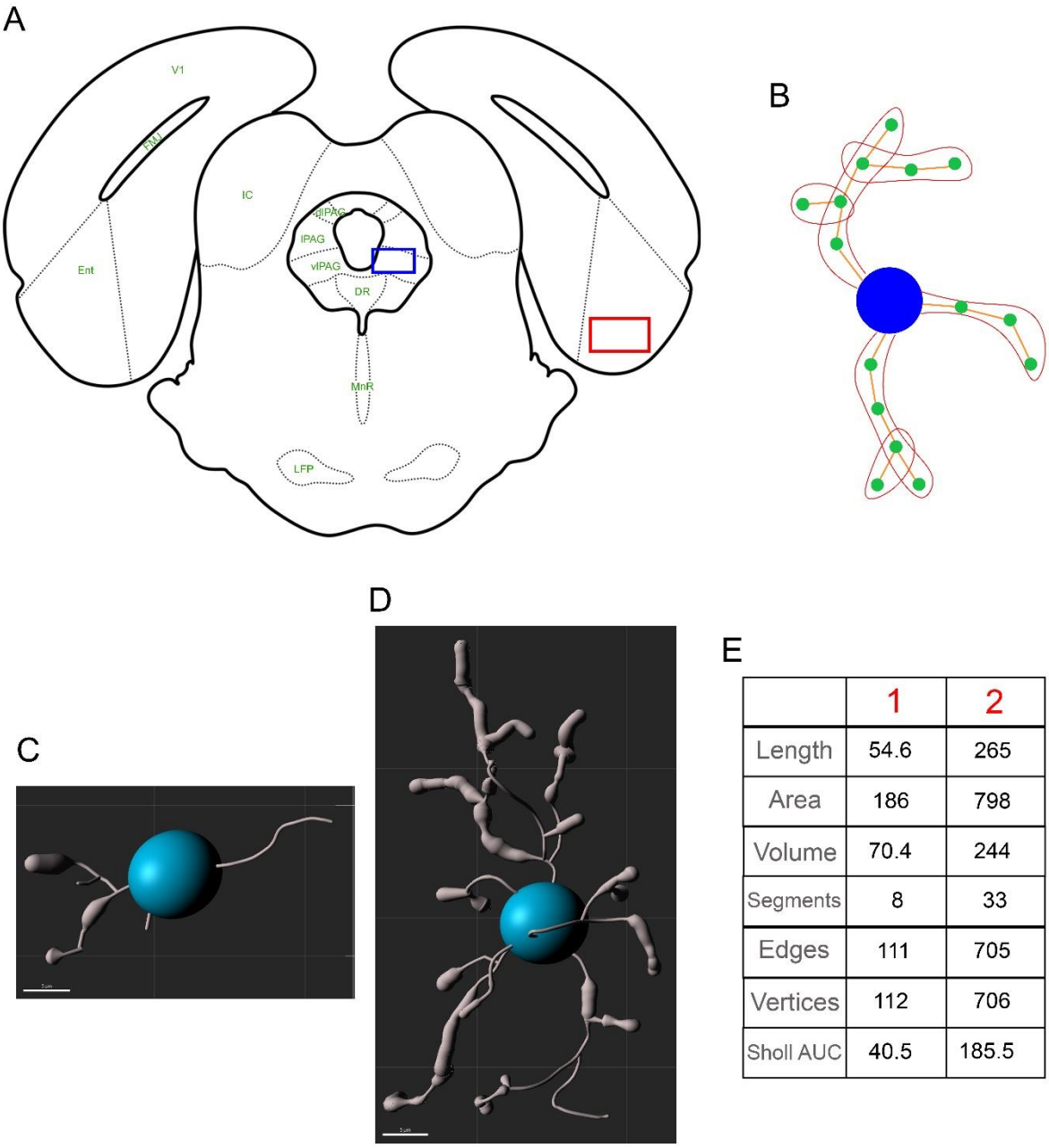
2.2.6 Microglia reconstructions

Microglial morphology was used as a metric for the central neuroinflammatory response to LPS. Microglial deramification and decreased complexity correlate with elevated cytokine production, suggesting that this morphometric glial state is representative of a proinflammatory microglia phenotype (Althammer et al., 2020). At eight hours post-LPS, rats were decapitated, brains were removed and drop-fixed in 4% paraformaldehyde for 24 hours, followed by 30% sucrose until sectioning (Harder et al., 2023). Fixed tissue was sectioned in a 1:6 series of 40-µm coronal sections with a Leica SM2010R microtome and stored in cryoprotectant at –20°C. Microglia were visualized using immunohistochemistry as previously described (Doyle and Murphy, 2017; Eidson and Murphy, 2013). Briefly, free-floating sections were rinsed thoroughly in potassium phosphate buffer solution (KPBS), incubated in 3% hydrogen peroxide at room temperature for 30 minutes, and then rinsed in KPBS. Sections were then incubated in 1:10,000 rabbit anti-Iba1 (Wako; 019-19741) diluted in KPBS with 1% Triton-X overnight at room temperature. Following rinses in KPBS, sections were incubated in 1:600 biotinylated donkey anti-rabbit (Jackson Immuno; 711-065-152) diluted in KPBS with 0.4% Triton-X for one hour at room temperature. Following KPBS rinses, sections were incubated in an Avidin/Biotin solution (PK-6100, Vector Labs) for one hour at room temperature, followed by KPBS and sodium acetate rinses. The sections were then incubated in a 3,3’-diaminobenzidine solution for 30 minutes, rinsed with sodium acetate and KPBS, and mounted onto slides. Slides were dehydrated using increasing concentrations of ethanol and cover-slipped. Microglial morphology was imaged in the ventrolateral PAG (level 4; 8.04 mm posterior to bregma; Figure 2-3A; blue box), given its central role in the regulation of opioid-induced immunosuppression.

As an anatomical control, microglial morphology was also analyzed in the entorhinal cortex (8.04 mm posterior to bregma; Figure 2-3A; red box). Like the PAG, the entorhinal cortex has dense μ-opioid receptor expression, but does not have a defined role in immune signaling. One to three sections per rat were imaged unilaterally at 40x on the Keyence BZ-X700 using the Z-stacking feature (1 μm steps). Microglia were then reconstructed using Imaris 10.0.0. Images were first converted into Z-stack TIFF files in FIJI, then converted to .ims files and opened in Imaris for preprocessing (inversion and background subtraction). Microglia morphology was then traced and analyzed using the filament creation wizard, followed by manual validation. A total of 1588 microglia were reconstructed and analyzed in the PAG (VEH M: 372 microglia from 6 rats; VEH F: 296 microglia from 4 rats; MOR M: 496 microglia from 8 rats; MOR F: 424 microglia from 7 rats), and a total of 266 microglia were reconstructed and analyzed in the entorhinal cortex (VEH M: 61 microglia, VEH F: 59 microglia, MOR M: 76 microglia, VEH F: 70 microglia).

Seven metrics were collected:

1. Length: total length of all edges in the microglia (μm).
2. Area: total area of each microglia (μm2).
3. Volume: total volume of each microglia (μm3).
4. Segments: total number of segments in the microglia.
5. Edges: total number of connections between vertices.
6. Vertices: total number of points connecting edges.
7. Sholl intersections: number of intersections on concentric spheres spaced 1 μm apart.



	1	2
Length	54.6	265
Area	186	798
Volume	70.4	244
Segments	8	33
Edges	111	705
Vertices	112	706
Sholl AUC	40.5	185.5

Figure 2-3. Location of vlPAG (A, red box) and entorhinal cortex (A, blue box) for analysis of microglial morphology. dlPAG = dorsolateral periaqueductal gray, DR = dorsal raphe, Ent = entorhinal cortex, FMJ = forceps major, IC = inferior colliculus, LFP = longitudinal fasciculus of the pons, lPAG = lateral periaqueductal gray, MnR = median raphe, V1 = primary visual cortex, vlPAG = ventrolateral periaqueductal gray. B. Example of segments, edges, and vertices. The blue circle represents the soma, orange lines represent edges, green circles represent vertices, and red outlines represent segments. Example of deramified (C) vs. ramified (D) microglia and their respective morphological values (E).

See Figure 2-3B for an example of segments, edges, and vertices. Figure 2-3 also shows an example of two microglia: one with deramified morphology (C) and one with ramified morphology (D), along with values of all seven metrics collected for each microglia (E). Deramified morphology is associated with smaller values, while ramified morphology leads to greater values on all seven metrics. Deramification correlates with cytokine production, suggesting that deramified microglia are in a functionally "active" state (Althammer et al., 2020). Sholl intersections were analyzed using area under the curve (AUC) such that smaller AUC values are representative of fewer Sholl intersections.

2.2.7 Gut permeability

Gut permeability was measured using oral administration of fluorescein-isothiocyanate-labeled dextran (FITC-dextran; molecular weight 4kDa; Sigma-Aldrich 46944). Adult male and female rats (P60) were fasted for four hours (beginning at nine AM). Following the fast, rats were orally dosed with 600 mg/kg of a 125 mg/mL solution of FITC-dextran using a 16g three-inch curved gavage needle with a 3mm ball. Four hours post-FITC-dextran, blood was collected from the saphenous vein into EDTA tubes and centrifuged (4°C, 3000g, 15 minutes) to separate plasma. Samples (100 µL) were transferred to a black-bottom 92 well plate, and relative fluorescent units (RFU) were read using a SpectraMax M2 plate reader (emission 530 nm, excitation 485 nm). Data were normalized within sexes to generate fold change of MOR vs. VEH rats.

2.2.8 Measurement of bacterial contact via anti-LPS antibody levels

Anti-LPS antibody levels were determined in adult male and female rats (P60) using ELISA. Blood was collected from the saphenous vein into uncoated microcentrifuge tubes,

allowed to clot for 30-60 minutes, then centrifuged (4°C, 3000g, 15 minutes) to generate serum. Plates were coated in-house using a 0.5% v/w LPS and carbonate-bicarbonate buffer solution (100 µL per well) and washed the following day using 0.05% goat serum and 0.01% TWEEN20 in PBS solution. The plate was then incubated at 37°C for one hour in the presence of 100 µL serum (per well; diluted 1:200). Following a wash, 100 µL of 0.1% v/v HRP-conjugated anti-rat IgG antibody was added to each well, incubated at 37°C for one hour and then washed again. The reaction product was visualized by adding 100 µL of SureBlue TMB (SeraCare; 5120-0075) to each well. After a five minute incubation in the dark at room temperature, 100 µL of TMB stop solution (SeraCare; 5150-0020) was added. Optical density was read using a Bio-rad iMark microplate reader at 450 nm.

2.2.9 Measurement of antibody production

To investigate whether any potential differences in anti-LPS antibodies were generalized or specific, levels of the three major subtypes of antibodies (IgG, IgA, and IgM) were quantified in adult male and female rats (P60) using ELISA. Blood was collected from the saphenous vein into EDTA tubes and centrifuged (4°C, 3000g, 15 minutes) to separate plasma. Total levels of IgG (ThermoFisher; 88-50490), IgA (ThermoFisher; 88-50480), and IgM (ThermoFisher; 88-50540) were assayed following the manufacturer's protocols. Optical density was read using a Bio-rad iMark microplate reader at 450 nm and compared to standard curves to calculate IgG, IgA, and IgM concentrations in ng/mL.

2.2.10 Experimental design and statistical analysis

Significant effects of sex, treatment, and time (where applicable) were assessed using two- or three-way mixed models or repeated measures mixed models; $p<0.05$ was considered

significant. As repeated measures ANOVA cannot handle missing values, data were analyzed by fitting mixed models with Greenhouse-Geisser correction as implemented in GraphPad Prism 9.1.0 (Motulsky, 2023). Tukey's or Sidak's post-hoc tests were conducted to determine significant mean differences between a priori specified groups. Due to the method of partitioning variance in linear mixed models, there is no universal method to calculate standardized effect sizes (e.g., η2 for ANOVA). Whenever possible, we report unstandardized effect sizes, which agrees with recommendations for effect size reporting (Pek and Flora, 2018), including the guidance of the American Psychological Association Task Force on Statistical Inference (Wilkinson, 1999).

As multiple microglia were reconstructed and analyzed from one rat, the assumption of independence was not met, and traditional statistical analyses could not be utilized. Therefore, hierarchical bootstrapping and permutation testing were used, which do not require independent observations (see Saravanan et al., 2020 for more details on hierarchical data in neuroscience and the application of bootstrapping for this type of data). Analyses were completed in R 4.2.3 using the package ClusterBootstrap (Deen and de Rooij, 2020). General linear models were utilized to create estimated means, and permutation testing was completed to investigate the robustness of the estimated cluster means. Data were visualized as a probability density function implemented in Excel 2019. Leftward shifts in population density function are indicative of increased activation in response to LPS.

All unstandardized effect sizes are reported as differences between means (MOR-VEH) ± standard error of the mean. Female rats used to generate offspring were randomly assigned to the MOR or VEH condition. All experiments included both male and female offspring. No

differences were observed between rats of different litters in the same drug exposure group (i.e., MOR and VEH); therefore, individual rats from the same litter across multiple litters served as a single cohort (4 VEH litters, 5 MOR litters). All analyses were completed blinded to the treatment group.

2.3 Results

2.3.1 24-hour LPS-induced fever

We first investigated the impact of perinatal morphine exposure on the response to LPS, focusing initially on fever and sickness behavior. After the prototypical spike in temperature due to handling stress (Machado et al., 2020), systemic administration of LPS induced a febrile response in both MOR and VEH groups (Figure 2-4A). Body temperature began to rise for both treatment groups at hour 2 and continued to increase from hours 3-6, with MOR males displaying a slower rise in temperature. Beginning at hour 6, body temperature began to decline for VEH males and females; in contrast, MOR males and females continued to increase and remained elevated throughout hours 7-8 before returning to baseline. By hour 11, body temperature was comparable for all rats, and by hour 16 all rats returned to baseline.

Overall, we observed a significant effect of treatment on LPS-induced fever [Time*Txt, $F(24,520)=2.290$, $p=0.0005$]; this difference was primarily driven by females [Txt*Sex, $F(1,37)=10.81$, $p=0.0022$], with MOR females maintaining a higher fever response for a longer period of time in comparison to VEH females (Figures 2-4B-C). We next analyzed two components of the fever response: time to maximum change in temperature and maximum change in temperature. For time to maximum change in temperature, although we observed no significant effect of treatment [Txt, $F(1,37)=3.508$, $p=0.0690$], MOR females took longer to

reach maximum fever (Figure 2-4D). Specifically, MOR female rats took 0.95 hours longer (VEH F vs MOR F, p=0.1811), shifting from an average time of 6.5 hours to 7.45 hours. No differences were noted for males (VEH M vs MOR M, p=0.9722). A significant main effect of treatment was observed in maximum change in temperature [Txt, F(1,35)=6.537, p=0.0151] (Figure 2-4E); this effect was also driven by females (VEH M vs. MOR M, p=0.9604; VEH F vs. MOR F, p=0.0318). The maximum temperature change for MOR F was 1.84°C vs. 1.46°C for VEH F, a 0.38°C difference, equivalent to a 26.2% greater temperature change.

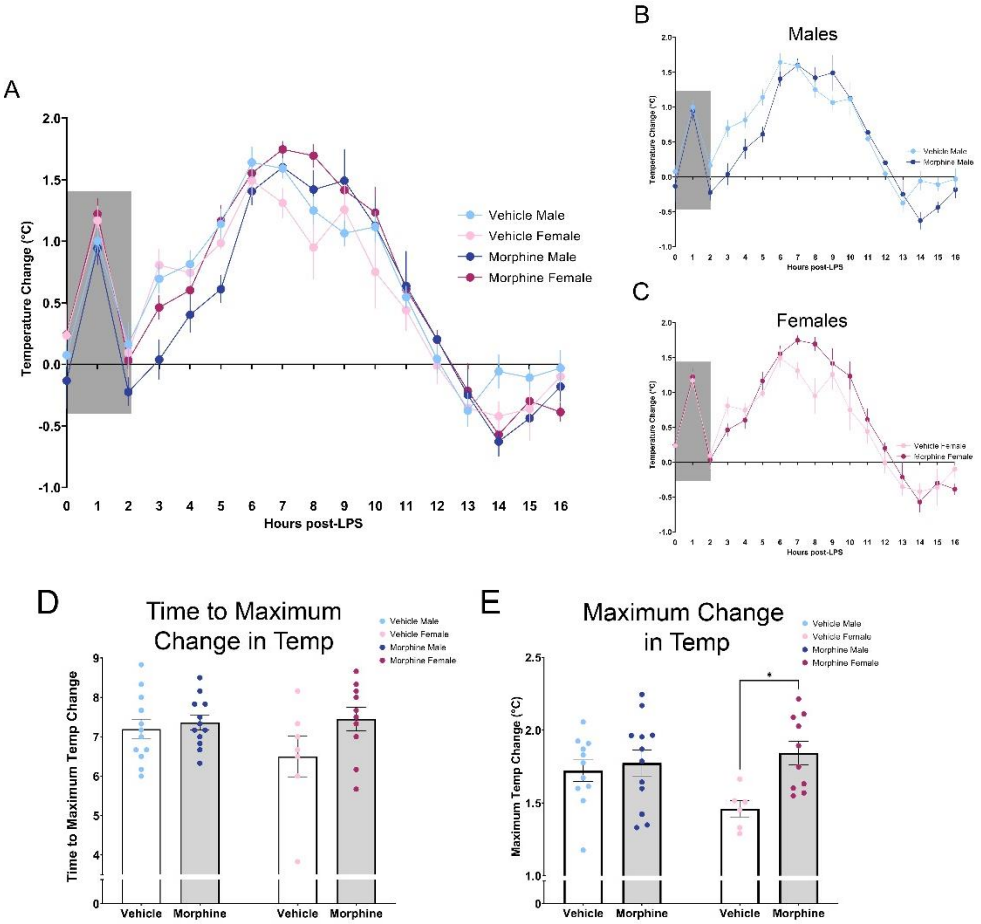


Figure 2-4. Perinatal morphine exposure leads to increased fever response to LPS. A. Fever arc from hours 0-16 post-LPS. B. MOR males showed a lower rise in body temperature from hours 3-6; temperature remained elevated vs. VEH males from hours 7-9. C. MOR females have elevated temperatures from hours 7-12. D. MOR males and females have delayed time to reach maximum fever. E. MOR males and females have increased maximum fever magnitude. $N_{VEH\ M}$ = 12, $N_{VEH\ F}$ = 7, $N_{MOR\ M}$ = 12, $N_{MOR\ F}$ = 10. * = significant at $p<0.05$.

Together, this data suggests that perinatal opioid exposure leads to increased febrile response to LPS. MOR males and females take longer to reach maximum fever and have a higher maximum fever, both of which were only significant in females.

2.3.2 24-hour sickness behavior

Similar to humans, rodents also display sickness-associated features in response to infections that function to conserve energy and aid in recovery and survival. To characterize this quantitatively, we utilized a Likert scale based on the presence/absence and severity of four physical attributes associated with sickness (Sotocinal et al., 2011). LPS induced robust sickness in VEH male rats and MOR male and female rats (Figure 2-5A); in contrast, VEH female rats showed minimal signs of sickness despite robust fever generation post-LPS [Time*Txt*Sex, $F(11,165)=2.170$, $p=0.0183$]. Overall, MOR females showed higher sickness scores vs. VEH females from hours 4-14; no significant differences were noted in MOR vs. VEH males (Figures 2-5B-C). MOR females also showed higher maximum sickness scores vs. VEH females [Txt, $F(1,15)=12.36$, $p=0.0031$; Sex, $F(1,15)=5.815$, $p=0.0292$], while males in both treatment groups showed comparable levels of sickness (Figure 2-5D). Importantly, sickness scores were positively correlated with maximum temperature change [$r(41)=0.4268$, $p=0.0043$], confirming that our sickness scale captured the relevant features associated with LPS-induced fever (Figure 2-5E).

Together, this data suggests that LPS-induced fever and sickness is increased in MOR male and female rats. We next examined potential mechanisms by which perinatal opioid exposure leads to an increased response to LPS.

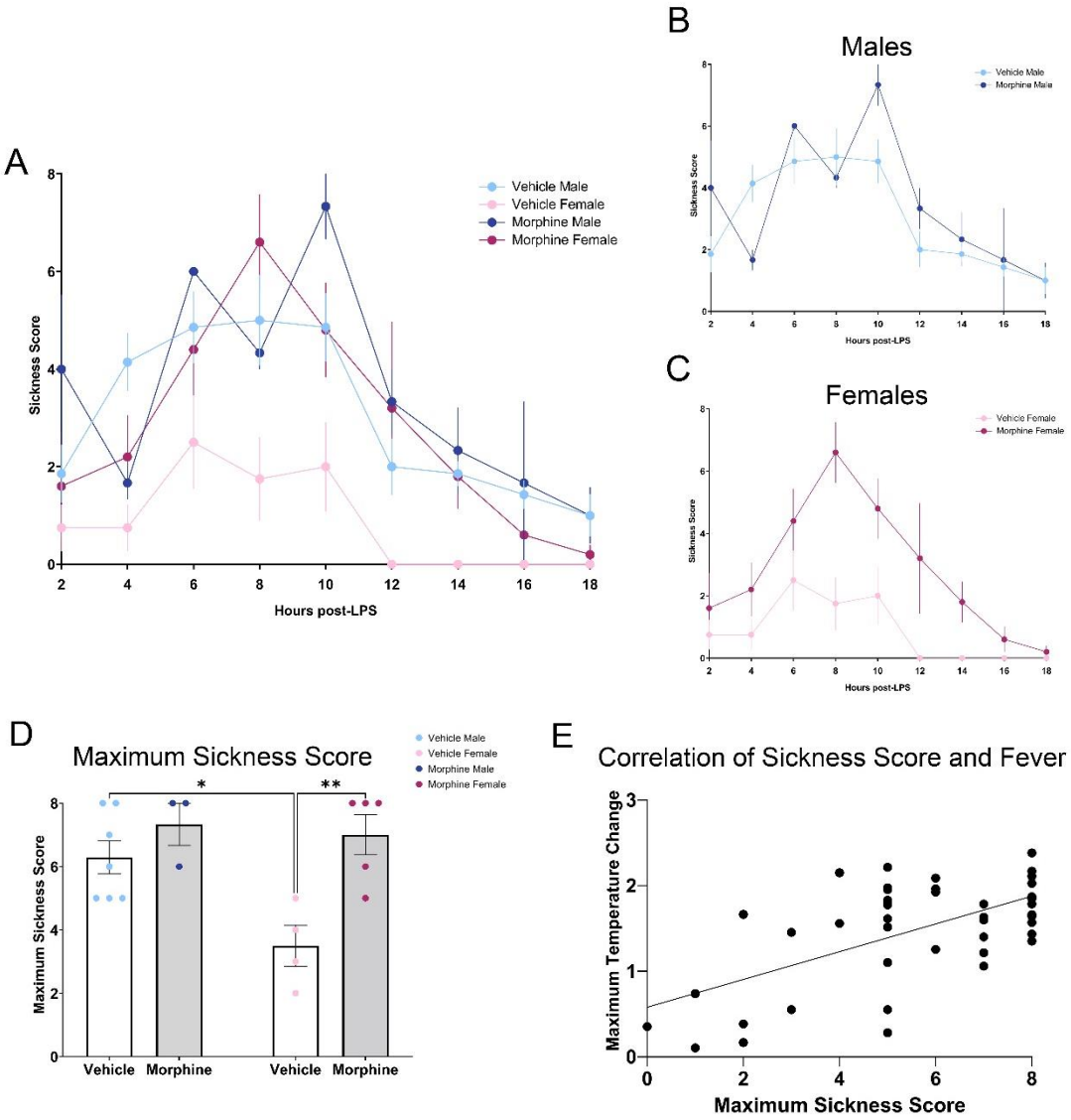


Figure 2-5. Perinatal morphine exposure leads to elevated sickness behavior in female rats. **A.** Sickness arc from hours 0-16. **B.** No difference was observed in males. **C.** MOR females have elevated sickness behavior. **D.** MOR females have elevated maximum sickness behavior. **E.** Maximum temperature and maximum sickness scores positively correlate. $N_{VEH\ M}$ = 9, $N_{VEH\ F}$ = 7, $N_{MOR\ M}$ = 12, $N_{MOR\ F}$ = 10. * = significant at $p<0.05$.

Based on the fever arc for VEH and MOR rats, we chose the 8 hour timepoint for analyses of central and peripheral immune measures. At this timepoint, both male and female MOR rats had significantly elevated body temperature [Txt, $F(1,37)=8.844$, $p=0.0052$], although only female rats reached statistical significance (VEH M vs. MOR M, $p=0.8196$; VEH F vs. MOR F,

p=0.0167) (Figure 2-6A). Body temperature at 8 hours post-LPS was 13.7% higher in MOR males (MOR M: 1.42±0.16°C; VEH M: 1.25±0.11°C) and 78.4% higher in MOR females (MOR F: 1.70±0.096°C; VEH F: 0.95±0.26°C). Sickness scores were similarly elevated, again only in MOR females [Txt*Sex, F(1,39)=7.69, p=0.0088] (Figure 2-6B). Specifically, the average sickness score for VEH females was 1.7; in contrast, the average sickness score for MOR females was 4.5, over 2.5 times greater [p=0.0325].

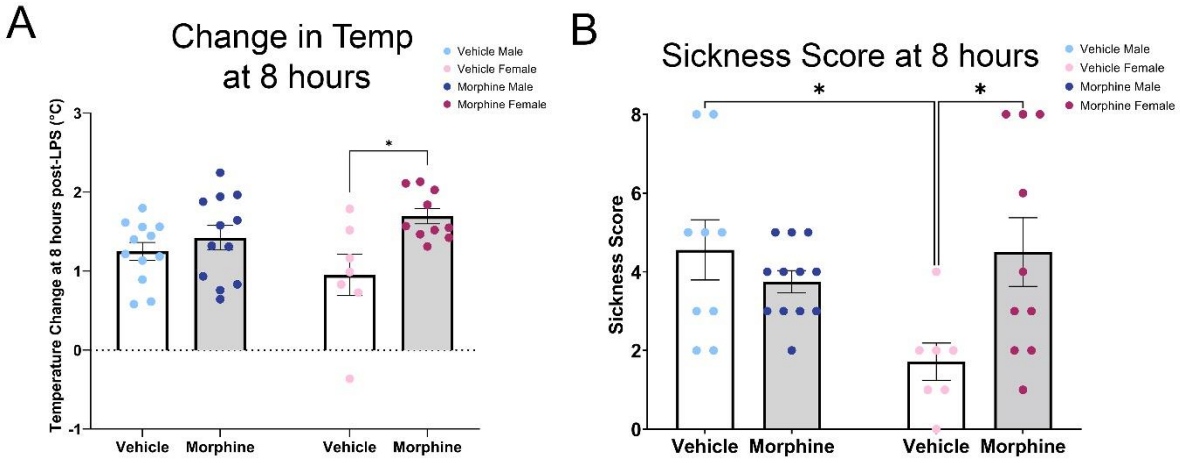


Figure 2-6. Perinatal morphine exposure leads to increased fever and sickness scores 8 hours post-LPS. **A.** MOR males and females have elevated body temperature at 8 hours post-LPS. **B.** MOR females have elevated sickness behavior at 8 hours post-LPS. $N_{VEH\ M}$ = 9-12, $N_{VEH\ F}$ = 7, $N_{MOR\ M}$ = 12, $N_{MOR\ F}$ = 10. * = significant at $p<0.05$.

2.3.3 Cytokine array

We first tested the hypothesis that perinatal exposure to morphine leads to long-term immune dysregulation, focusing initially on plasma cytokine levels, which constitute a major component of an organism's response to LPS and infection, and are responsible for fever induction. Our analysis focused on four cytokines implicated in inflammation and response to infection: IFN-γ, IL-1α, IL-1β, and IL-10. A significant effect of treatment was only observed in one cytokine, IL-1α [Txt, F(1,18)=10.96, p=0.0039] (Figure 2-7A), with concentrations three

times greater in MOR vs. VEH rats. IL-1α acts primarily in the hypothalamus to generate fever, suggesting that the elevated level of this cytokine may be the primary driver of the elevated fever observed in MOR rats. Given the variability in the levels of IL-1α in MOR rats, we next investigated if IL-1α levels were correlated with fever response. However, IL-1α was not correlated to temperature change 8 hours post-LPS (Figure 2-7B; r(22)=0.3032, p=0.1701). We also investigated levels of IFN-γ, IL-1β, and IL-10; however, no significant treatment differences were observed (Figure 2-7C-E).

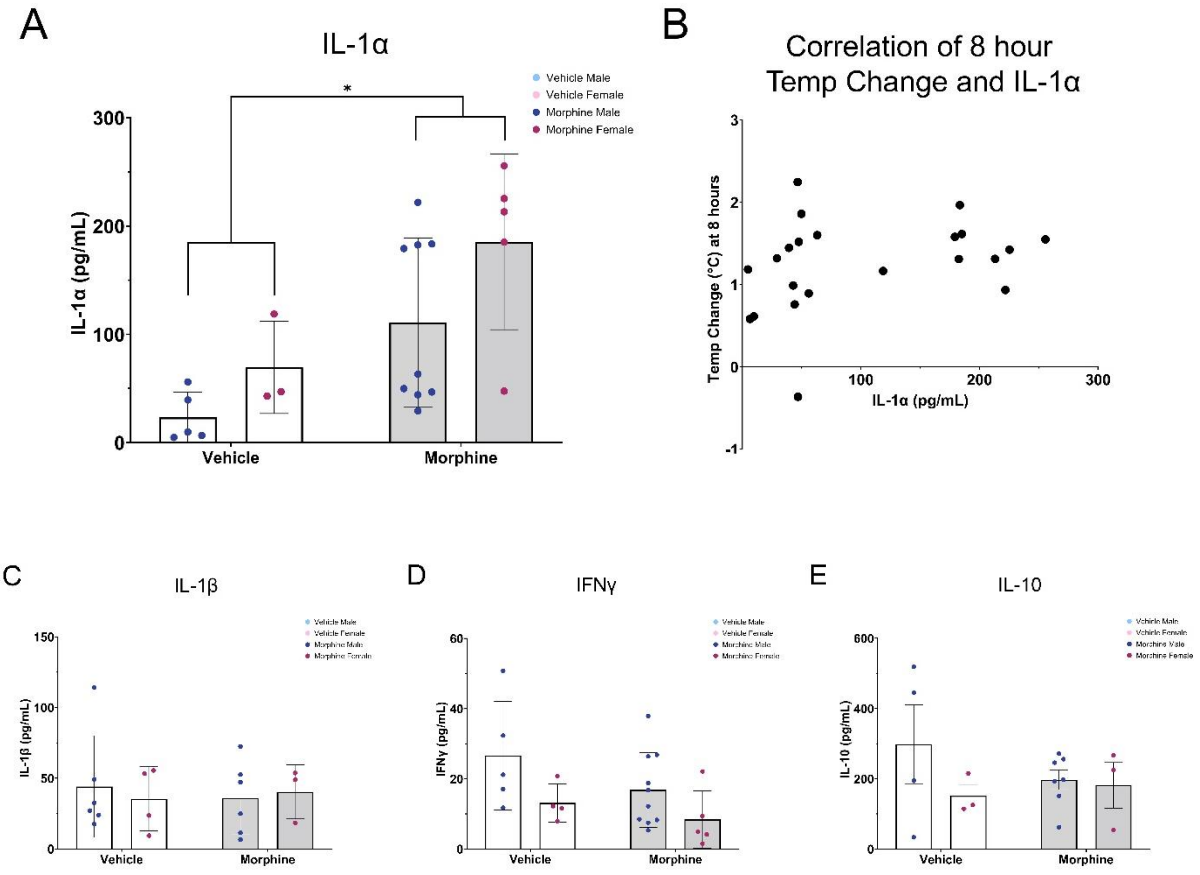


Figure 2-7. Alterations in cytokine levels post-LPS. **A.** Elevated IL-1α levels in MOR rats. **B.** No correlation of IL-1α with temperature change 8 hours post-LPS. **C.** No changes in IL-1β. **D.** No changes in IFNγ. **E.** No changes in IL-10. $N_{VEH\ M}$ = 4-6, $N_{VEH\ F}$ = 3-4, $N_{MOR\ M}$ = 6-10, $N_{MOR\ F}$ = 3-5. * = significant at $p<0.05$.

2.3.4 Microglia reconstructions.

Although morphine is typically considered immunosuppressive in the periphery (decreased natural killer cell cytotoxicity, reduced macrophage phagocytosis, and altered proinflammatory cytokine production), centrally, morphine promotes microglial reactivity and initiates cytokine release in a TLR4-dependent manner (Eidson et al., 2017; Wang et al., 2012). Thus, we next examined if perinatal morphine alters the microglial response to LPS.

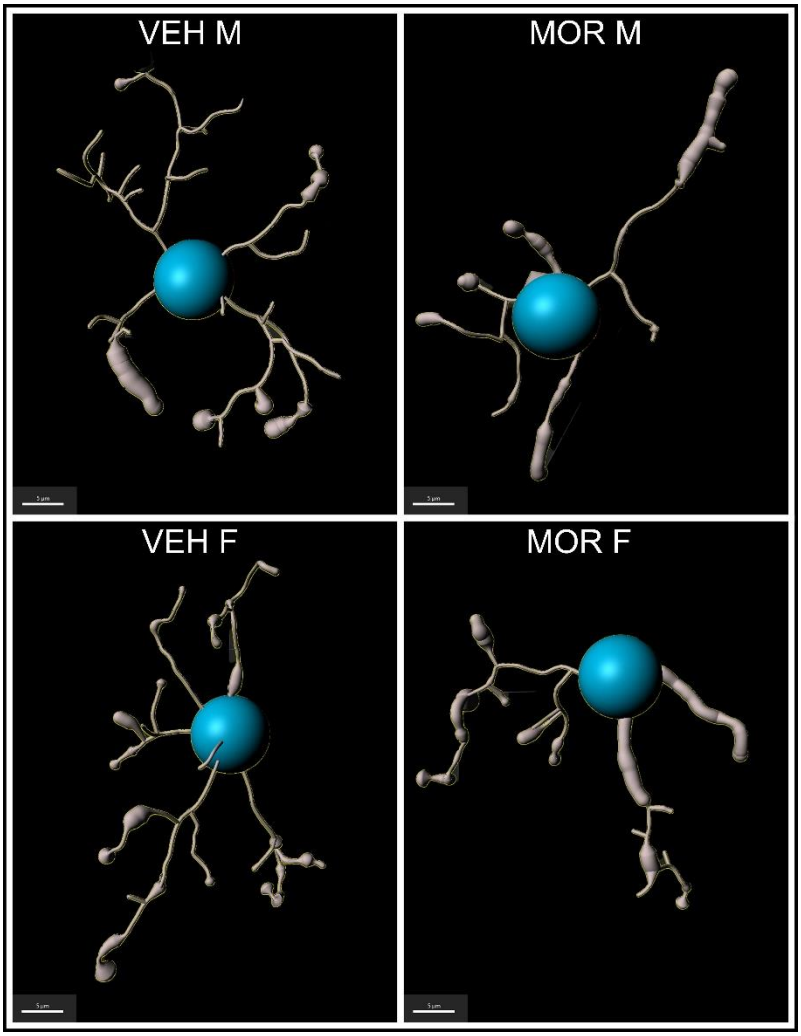


Figure 2-8. Representative traces of microglia from the PAG of VEH M, VEH F, MOR M, and MOR F.

Microglia respond to LPS by transforming to a more "activated" and deramified morphology, reflected as smaller size and less complex structure. Thus, we predicted that microglia would show increased deramified morphology in MOR rats. Microglia morphology was reconstructed, and seven metrics were analyzed: length, area, and volume; segments, edges, and vertices; and Sholl intersections (see Figure 2-8 for representative microglia traces from all four groups). In all metrics, smaller measurements represent deramified and “activation-associated” morphology.

We first analyzed microglia size (length, area, and volume) and observed a leftward population shift for all three measures in MOR rats independent of sex (Figure 2-9A-C). Hierarchical bootstrapping followed by permutation testing identified a significant difference in mean microglial length of -17.04 µm (-9.6%) and -18.82 µm (-10%) for MOR male and female rats, respectively, in comparison to VEH rats. Similar results were observed for both area and volume: for area, mean differences of -49.64 µm2 (-9.8%) and -25.4 µm2 (-5.1%) were observed in MOR male and female rats; for volume, MOR male and female rats had mean differences of -19.33 µm3 (-11.2%) and -3.1 µm3 (-2.0%). See Table 2-1 for descriptive statistics for all metrics analyzed. Overall, the observed leftward shift in microglia size for MOR rats suggests that POE increases microglial activation-associated morphology (i.e., deramification) in response to LPS.

We also analyzed additional measures of size and complexity, including the number of segments, edges, and vertices present per microglia (Figure 2-9D-F). Consistent with the results observed for size, all measures were reduced in MOR vs. VEH rats. Mean differences of -3.25 (-12.8%) and -4.8 segments (-18.5%) were observed in MOR male and female microglia. For edges, mean differences of -23.86 (-5.9%) and -19.02 (-4.5%) were noted for MOR vs. VEH rats,

and for vertices mean differences of -23.86 (-5.9%) and -19.03 (-4.5%) were observed. Overall, MOR male and female rats again showed left-shifted populations of microglia size and complexity, consistent with increased activation.

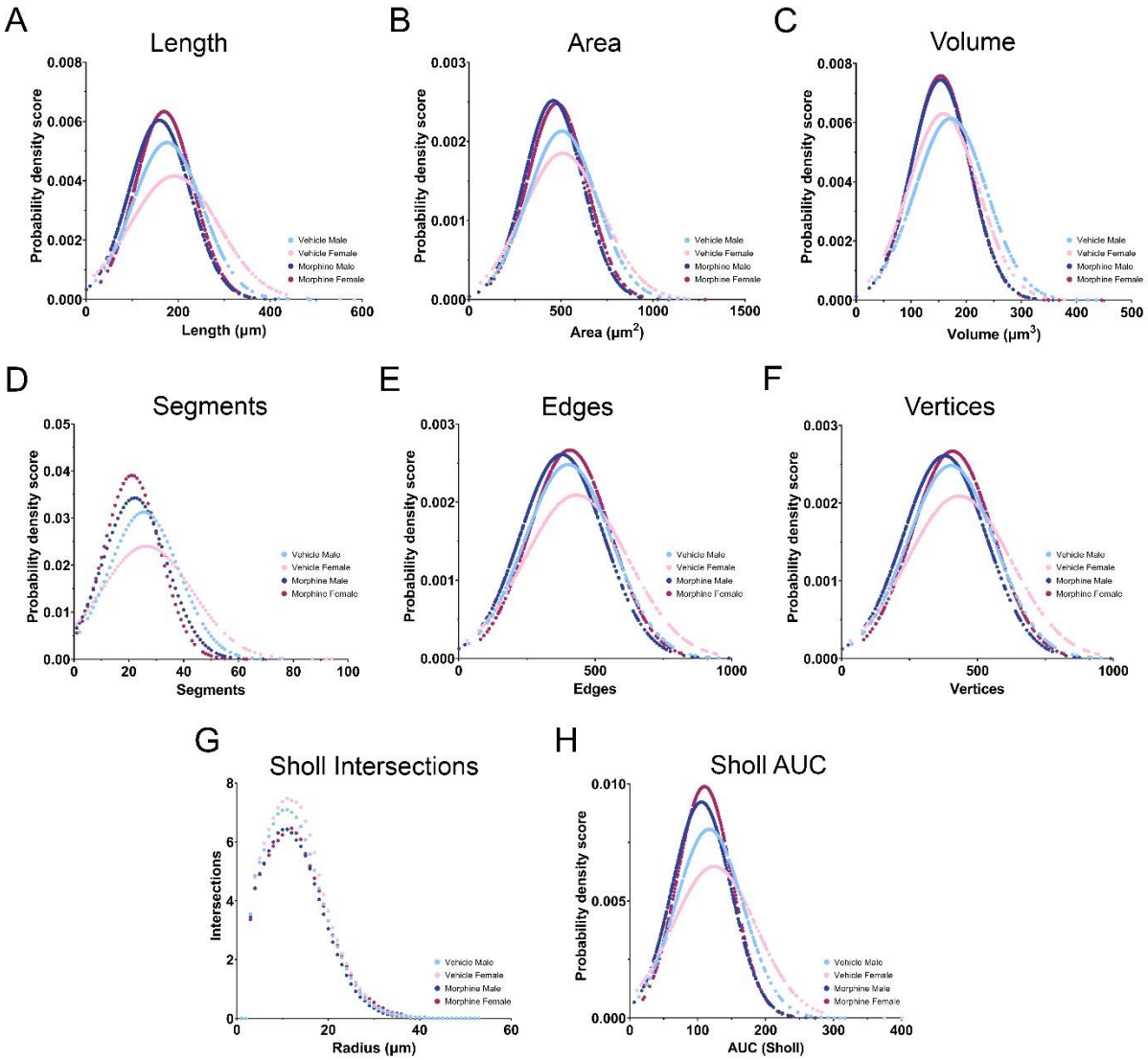


Figure 2-9. Microglia activation 8 hours post-LPS. **A-C.** Length, area, and volume are all decreased in MOR male and female rats. **D-F.** The number of segments, edges, and vertices is decreased in MOR male and female rats. **G.** MOR male and female rats have fewer intersections across the entire Sholl radius. **H.** Sholl AUC is decreased in MOR male and female rats. $N_{VEH\ M}$ = 372 microglia, $N_{VEH\ F}$ = 296 microglia, $N_{MOR\ M}$ = 496 microglia, $N_{MOR\ F}$ = 424 microglia.

We next analyzed Sholl intersections, a classic metric of microglial activation. Comparison of the number of intersections per concentric circle (1 μm apart) showed lower intersections across the entire range for MOR male and female microglia, consistent with deramification (Figure 2-9G). To analyze Sholl intersections via hierarchical bootstrapping, we utilized area under the curve (AUC) of individual microglia. Overall, MOR male and female microglia had lower Sholl AUC values (Figure 2-9H), representing fewer intersections and suggesting increased activation.

Together, all seven metrics of activation-associated morphology in microglia were significantly lower for MOR male and female rats vs. VEH controls, suggesting that perinatal opioid exposure potentiated the microglial response to LPS.

To determine whether the increased microglial activation observed in the PAG of MOR rats was specific to the PAG, we next examined microglial morphology in the entorhinal cortex, a region with high μ-opioid receptor expression but no known role in immunity. Surprisingly, results for the entorhinal cortex were similar to what was observed in the PAG: in all seven metrics, MOR male and female rats display increased activation-associated microglial morphology (see Table 2-2 for a summary of these results). This suggests that increased microglial activation in response to LPS may be a generalized response in brain regions with high μ-opioid receptor expression (PAG and entorhinal cortex) for male and female rats perinatally exposed to morphine.

Thus far, we have reported that perinatal opioid exposure potentiates the response to LPS, as indicated by increased fever and sickness, elevated levels of IL-1α and increased microglial activation. We next examined if these differences were related to basal differences in

immune function, such that MOR rats are less able to launch an appropriate immune response following exposure to antigens or pathogens. We first investigated gut permeability using FITC-dextran dissemination into the bloodstream. Bacteria in the gut are a major source of immune system stimulation and training, and increased gut permeability would promote increased bacterial contact with the immune cells in the lamina propria, leading to differential immune system development (Kaczmarczyk et al., 2021). In addition, opioids are known to slow gut peristalsis, which is associated with increased gut permeability (Akbarali and Dewey, 2019).

2.3.5 Analysis of gut permeability using FITC-dextran

Due to differences in basal levels between experimental rounds, data were normalized to the mean of the VEH group to generate fold changes for each sex. Overall, no significant differences in gut permeability were noted (Figure 2-10A-B). However, MOR females had an average fold change of 1.47 relative to VEH females, representing a 47.1% increase in gut permeability [unpaired T test; $t(16)=2.038$, $p=0.0585$]; no differences were observed for males [fold change 0.79; unpaired T test; $t(14)=1.505$, $p=0.1546$]. This suggests that exposure to morphine *in utero* leads to long-term increases in gut permeability for female rats, which may alter immune system development and impact the response to immune stimulators.

2.3.6 Measurement of bacterial contact via anti-LPS antibody levels

To confirm our observed, albeit non-significant, increases in gut permeability in MOR female rats, we next quantified levels of anti-LPS antibodies. Increased gut permeability would allow for increased bacterial dissemination into the lamina propria; as LPS is one of the major antigens utilized by the immune system to recognize bacteria, we predicted that levels of anti-LPS antibodies would be similarly elevated in MOR females. We observed no significant effect

of sex, so males and females were combined to increase power. In contrast to our predicted increase, we observed a significant decrease in anti-LPS antibodies in MOR rats [t(34)=2.191, p=0.0354] (Figure 2-10C). Anti-LPS antibody levels were, on average, 27.1% lower in MOR vs. VEH rats. As the increased gut permeability observed in MOR females is in conflict with the observed decrease in anti-LPS antibodies, we next measured levels of antibody classes IgG, IgA, and IgM to identify potential deficits in antibody production as an alternative explanation for decreased anti-LPS antibody levels.

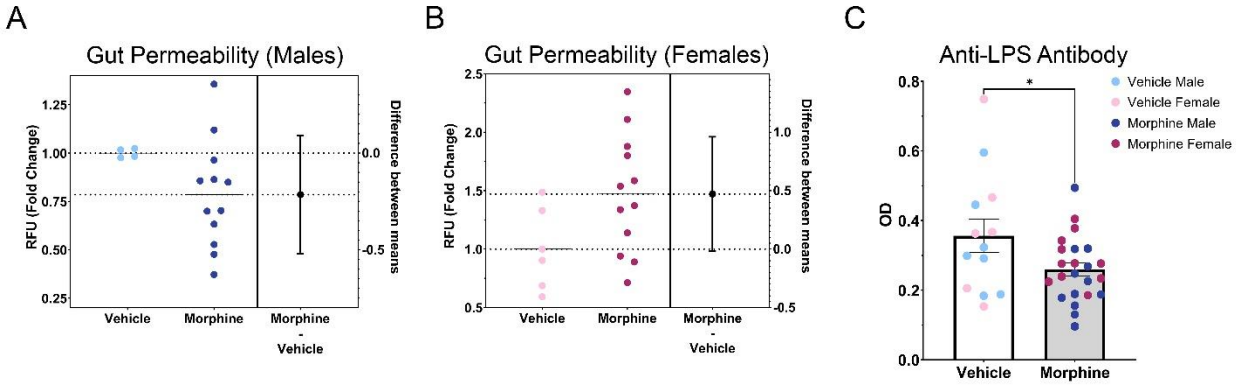


Figure 2-10. MOR female rats have increased gut permeability; however, both male and female MOR rats have decreased anti-LPS antibody levels. **A.** No differences in gut permeability were found between males. **B.** MOR females have elevated gut permeability. **C.** MOR males and females have decreased anti-LPS antibody levels, despite elevated gut permeability seen in females. $N_{VEH\ M}$ =4-5, $N_{VEH\ F}$ = 5-6, $N_{MOR\ M}$ = 8-12, $N_{MOR\ F}$ = 10-12. * = significant at $p<0.05$.

2.3.7 Measurement of antibody production

We first analyzed IgG, the most common and abundant antibody subtype, and a primary mechanism to target microbes for phagocytosis. Our analysis identified a significant interaction of treatment and sex [F(1,25)=9.525, p=0.0049] (Figure 2-11A). Specifically, we noted a 56% reduction in IgG production in MOR females (VEH F: 7.20*107±1.84*106 ng/mL; MOR F: 3.14*107 ±6.08*106 ng/mL; p= 0.0002). Similar mean differences were observed in MOR males,

with a reduction of 30% (VEH M: 2.50*107±6.12*106 ng/mL; MOR M: 1.75*107±2.31*106 ng/mL; p=0.6950). We also report that IgG levels were three times higher in VEH females vs. VEH males (p<0.0001), consistent with results showing that females typically have a more robust antibody response and higher baseline immunoglobulin levels (Klein and Flanagan, 2016).

We next analyzed IgA, which is involved in mucosal immunity and defense against oral or respiratory pathogens. There was no significant effect of sex, so males and females were collapsed to increase power. IgA levels were significantly higher in MOR rats [t(23)=2.288, p=0.0317] than VEH rats (VEH: 48669±12854 ng/mL; MOR: 153596±30551 ng/mL; 215% increase; Figure 2-11B).

Last, we analyzed IgM, a stimulator of the classical complement system. Two-way ANOVA identified a significant interaction of treatment and sex [F(1,28)=5.974, p=0.0211] (Figure 2-11C). In MOR females, IgM levels were reduced by 45.1% (VEH F: 283180±71072.20 ng/mL; MOR F: 155501.25±25274.86 ng/mL, p=0.0853), while a non-significant increase was noted in males [26.8% increase; VEH M: 135285.71±29888.72 ng/mL; MOR M: 171505±20246.52 ng/mL; p=0.8334]. We again noted that VEH females had IgM levels twice that of VEH males [p=0.0432].

Together, this data suggests that perinatal opioid exposure results in generalized deficits in antibody production, observed both in anti-LPS antibodies and IgG and IgM (females only) levels. Interestingly, a significant increase in IgA levels was noted for MOR rats, potentially related to alterations in enteric immunity and gut permeability.

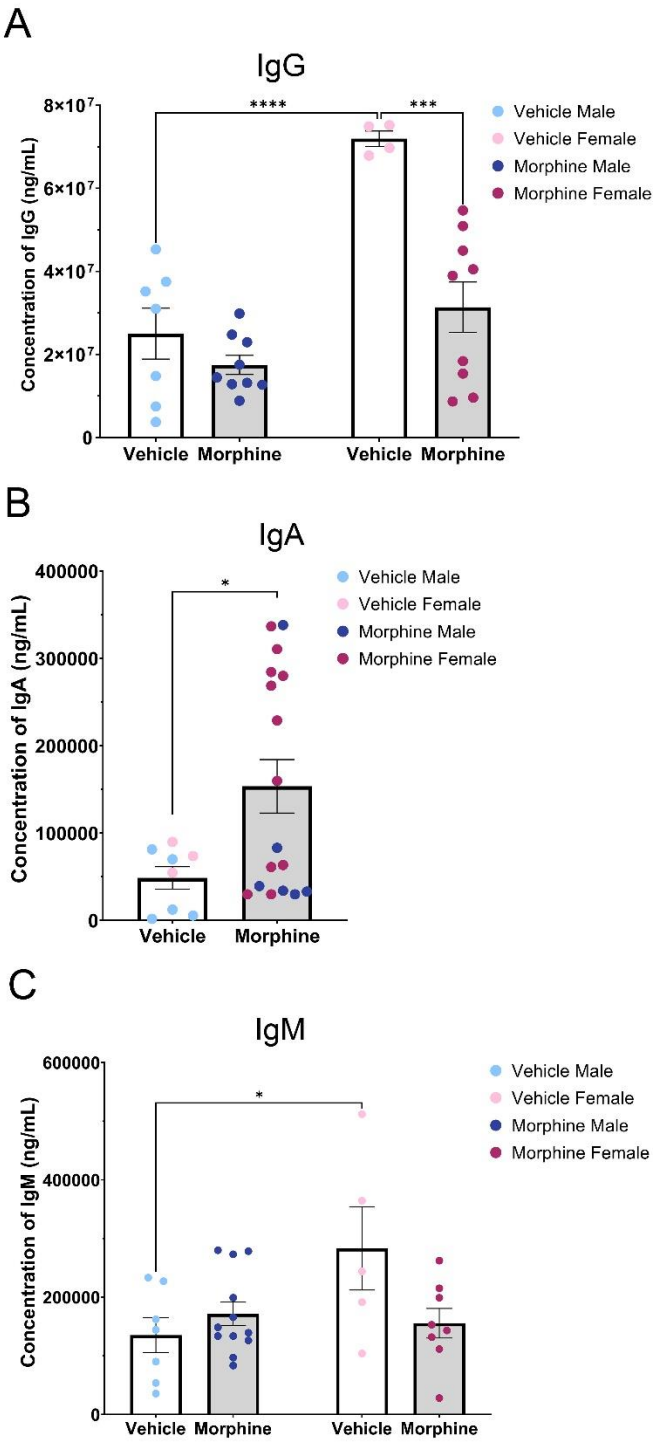


Figure 2-11. Perinatal opioid exposure alters antibody levels. **A.** MOR females have decreased levels of IgG. **B.** MOR male and female rats have elevated IgA antibody levels. **C.** MOR female rats trend toward lower levels of IgM. $N_{VEH\ M}$ = 5-7, $N_{VEH\ F}$ = 3-5, $N_{MOR\ M}$ = 6-12, $N_{MOR\ F}$ = 8-11. * = significant at $p<0.05$.

Results from measurements of baseline immune functioning in MOR vs. VEH rats suggest that *in utero* exposure to morphine leads to long-term alterations in immune system activity, including increased gut permeability and decreased antibody production. These differences may explain the increased fever and sickness responses to LPS and altered cytokine levels and microglial activation post-LPS. See Table 2-3 for a summary of the results.

2.4 Discussion

Previous clinical studies indicate that infants exposed to opioids *in utero* are at a higher risk of infection and hospitalization (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017). However, to date, the underlying mechanism by which perinatal opioid exposure leads to this increased risk is unknown. The present study was designed to address this gap, and, in particular, to characterize the physical and immunological response to LPS using a preclinical model of perinatal opioid exposure. We hypothesized that perinatal opioid exposure would compromise gut permeability and antibody production, consistent with the known immunosuppressive effects of chronic opioids in adult humans and rodents. Furthermore, we hypothesized that these effects would alter the response to an immune stimulator, LPS, observed as changes in fever, sickness, and markers (peripheral and central) of inflammation.

We first investigated the effects of perinatal opioid exposure on the response to an experimental model of Gram-negative bacterial infection, LPS. Our studies revealed that MOR rats responded with elevated fever and sickness following LPS administration vs. their VEH counterparts. Although increased fever and sickness may seem contradictory to the well-characterized immunosuppressive effects of opioids, fever is widespread among immunosuppressed patients and may even be the only symptom of infection (Pizzo, 1999).

Thus, while healthy individuals can regulate their immune system properly, immunosuppressed patients, particularly those who are neutropenic (i.e., with low levels of neutrophils), frequently show rapid rises in core body temperature and quickly proceed to sepsis. Indeed, previous studies investigating the response to LPS in rats made leukopenic (i.e., with a low number of circulating leukocytes) via chemotherapy have reported increased fever response to LPS, along with alterations in cytokine production (Miñano et al., 2004; Tavares et al., 2006, 2005). Interestingly, the time course and magnitude of fever in these studies are similar to what is observed in the present study for MOR rats. This, along with our observed deficits in antibody production, suggests that perinatal opioid exposure may produce neutropenia and/or leukopenia.

In addition to the febrile response, the majority of rats that received LPS also displayed physical characteristics of sickness. Specifically, male rats and MOR female rats looked sick, with ears flattened back, eyes tightened, nose flattened, and piloerect fur. Surprisingly, female VEH rats displayed very few physical attributes of sickness, despite a robust fever response. No difference in sickness score was noted for MOR vs. VEH males, perhaps due to a potential ceiling effect, given the high sickness scores observed in VEH males. Male rodents generally exhibit more severe sickness behavior following LPS, including anorexia (Kuo, 2016; Pitychoutis et al., 2009), huddling, piloerection, ptosis, lethargy (Cai et al., 2016), and reduced locomotion (Yee and Prendergast, 2010); however see (Pitychoutis et al., 2009). For the present studies, we chose to use a low dose of LPS that would enable us to observe either an increase or decrease in sickness score. Use of even lower doses of LPS may elucidate whether our observed sex

difference in VEH rats is due to increased sensitivity to the sickness-promoting effects of LPS in males.

Peripheral cytokine levels were also altered in response to LPS treatment. Elevation of IL-1α levels in MOR male and female rats at eight hours post-LPS likely contributes to the potentiated fever response seen in MOR rats, as IL-1α binds to IL-1R in the anterior and paraventricular nucleus of the hypothalamus and initiates a downstream cascade ending in synthesis of the pyrogen prostaglandin E2 (Cartmell et al., 1999), which increases body temperature via brown adipose tissue metabolism and vasoconstriction. Previous studies have indicated that acute morphine, given in conjunction with LPS, led to an increase in IL-1α levels in the brain (Roy et al., 1999), potentially through synergy with TLR4 (Eidson et al., 2017). Other models of perinatal opioid exposure have reported increases in adult levels of TLR4 and MyD88 (Jantzie et al., 2019; Smith et al., 2022). As IL-1α is one of the primary cytokines released after TLR4 stimulation via NF-κB signaling, this provides a potential mechanism by which perinatal morphine exposure increases IL-1α levels. We also noted a high degree of variability in IL-1α levels; surprisingly, this was not related to maximum temperature induced by LPS, but rather, this may be associated with the degree of immunosuppression in MOR rats, including elevations of TLR4 and/or MyD88.

The current study also identified increased microglial activation in the ventrolateral periaqueductal gray in male and female MOR rats following LPS treatment, suggesting that POE leads to long-term changes in microglial reactivity. While opioid exposure typically promotes peripheral immunosuppression, previous studies have shown that microglia are generally activated by morphine in a TLR4-dependent manner (Doyle et al., 2017). Increased microglial

activation likely contributed to the increased fever and sickness behavior following LPS, as activated microglia release cytokines that act in the hypothalamus to promote fever and sickness behavior. In the present study, only peripheral cytokines were assessed. Here, we observed a significant increase in IL-1α; however, plasma cytokine levels are not always representative of local brain region concentrations, so future studies should investigate local cytokine levels in the hypothalamus and PAG. We also analyzed microglial phenotype in the entorhinal cortex to determine if the observed increase in microglial activation was specific for the PAG, or a more widespread phenomenon. Surprisingly, microglia in the entorhinal cortex of both male and female rats showed increased activation-associated morphology. The entorhinal cortex is primarily associated with memory formation and learning (Maass et al., 2015) and to date has not been implicated in immune signaling. As both the PAG and entorhinal cortex have high levels of μ-opioid receptor expression, this suggests that perinatal morphine may act on μ-opioid receptors in multiple regions of the CNS to decrease the threshold for microglial reactivity, potentially through morphine's action as a developmental stressor (Carloni et al., 2021). Future studies should investigate microglial reactivity in additional brain regions, both with and without μ-opioid receptor expression.

Given the observed changes in LPS-induced fever and sickness in MOR rats, we next investigated if these changes were due to alterations in basal peripheral immune function or specifically a result of immune stimulation. We hypothesized that morphine exposure would promote gut permeability and increase the level of anti-LPS antibodies due to increased bacterial dissemination into circulation and that these together would potentiate the response to LPS. Here, we report that MOR rats displayed increased gut permeability, but surprisingly

produced fewer anti-LPS antibodies. The reduced level of antibodies was not specific to anti-LPS, as MOR rats had lower levels of the antibody subtypes IgG and IgM (females only), suggesting an overall deficit in antibody production that would predispose these rats to infection. Interestingly, we also observed significantly increased IgA antibody levels in MOR male and female rats. This may be related to morphine's effects on the gut, including gut permeability, as IgA is primarily involved in mucosal immunity and response to oral pathogens. Together, our observed deficits in IgG and IgM are consistent with clinical data reporting increased hospitalization rates for infection in children born with *in utero* opioid exposure (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017). The cause of antibody production deficits, including a decrease in the number of antigen-presenting cells and/or B lymphocytes involved in antibody production or the ability of these cells to produce antibodies in response to antigen stimulation, warrants further investigation. As increased gut permeability is also associated with changes in gut microbiota composition, the impact of perinatal morphine exposure on gut microbiota composition and its relationship to other immune parameters should also be examined.

Overall, our data suggests that rats perinatally exposed to morphine have an immunosuppressed phenotype (specifically increased gut permeability and deficits in antibody production) that may increase the susceptibility of the immune system to a pathogen or immune challenge, consistent with our finding that MOR rats show a potentiated fever and sickness response to LPS (see Figure 2-12 for a summary of the results). These results provide further evidence that exposure to opioids *in utero* leads to long-term immune deficits. As the number of infants born to mothers using opioids during pregnancy continues to rise,

determining the underlying mechanism whereby these infants are more vulnerable to potential pathogen exposure is critical.

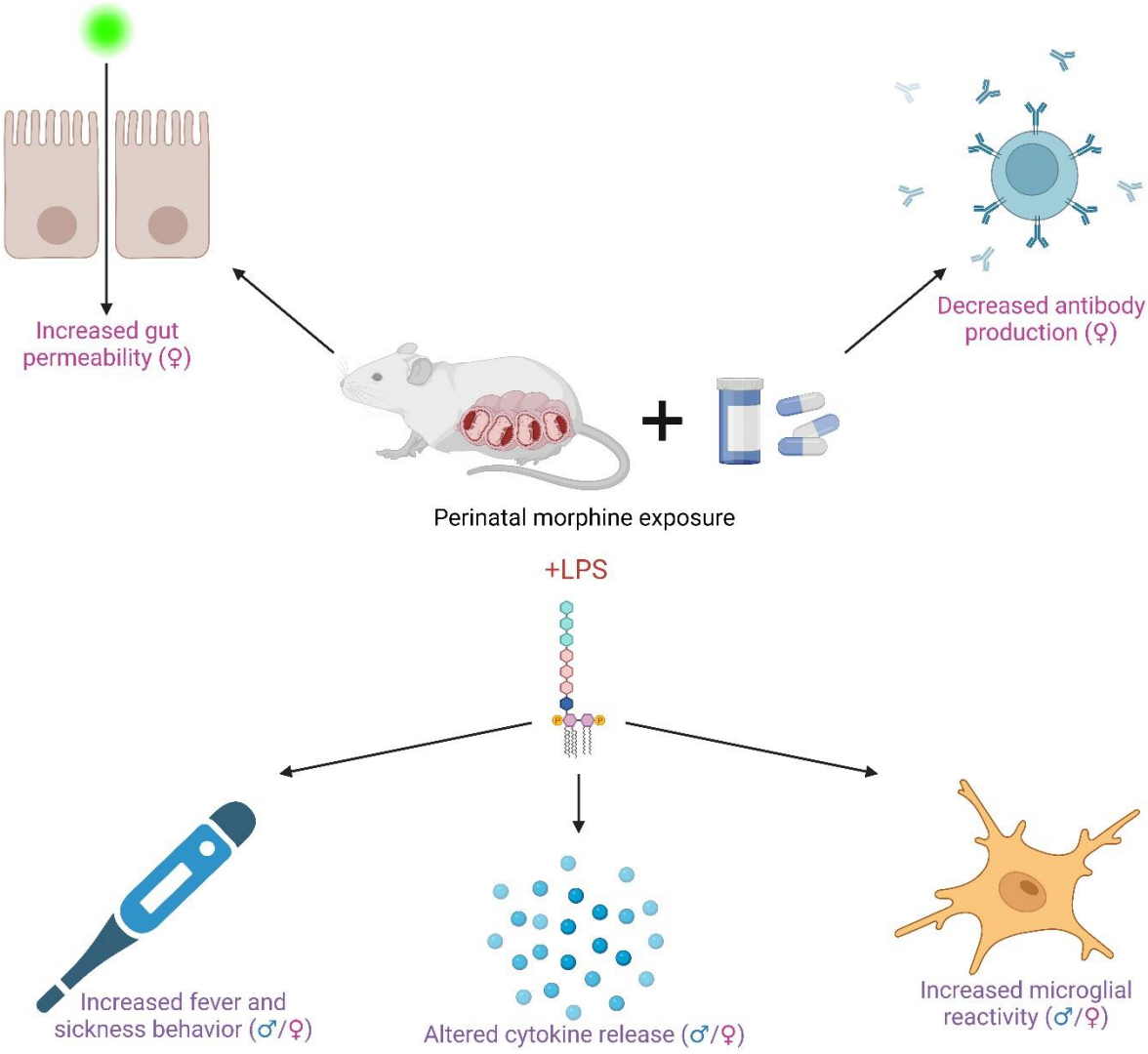


Figure 2-12. Summary of results. Created with BioRender.com.

	Males				Females			
	VEH Mean±SD MOR Mean±SD	Percent Change	Welch's t value	p value	VEH Mean±SD MOR Mean±SD	Percent Change	Welch's t value	p value
Length	176.84±12.51 µM 159.8±13.14 µM	-9.6%	-3.40	<0.001*	188.36±26.58 µM 169.54±13.76 µM	-10.0%	-3.45	0.001*
Area	507.68±32.97 µM^2 458.04±33.96 µM^2	-9.8%	-3.96	<0.001*	502.62±61.65 µM^2 477.22±36.88 µM^2	-5.1	-2.17	0.032*
Volume	172.26±11.97 µM^3 152.93±12.19 µM^3	-11.2%	-4.51	<0.001*	156.42±18.28 µM^3 153.32±13.04 µM^3	-2.0%	-1.06	0.3
Segments	25.41±2.07 22.16±2.37	-12.8%	-3.97	<0.001*	25.89±4.41 21.09±2.27	-18.5%	-4.89	<0.001*
Edges	402.21±21.75 378.35±24.61	-5.9%	-2.15	0.034*	425.84±46.28 406.82±26.83	-4.5%	-1.84	0.072
Vertices	403.21±21.75 379.35±24.61	-5.9%	-2.15	0.034*	426.85±46.28 407.82±26.83	-4.5%	-1.84	0.072
Sholl AUC	117.13±7.66 105.63±8.24	-9.8%	-3.56	<0.001*	122.55±16.14 109.98±8.57	-10.3%	-3.49	<0.001*

Table 2-1. Summary of alterations in microglial morphology in the periaqueductal gray. % represent percent change from sex-matched VEH controls. * = significant at p<0.05.

	MOR M			MOR F		
	Percent Change	Welch's t value	p value	Percent Change	Welch's t value	p value
Length	-18.0%	-3.16	0.003*	-22.5%	-3.67	<0.001*
Area	-25.2%	-5.27	<0.001*	-15.4	-2.76	0.007*
Volume	-25.8%	-5.75	<0.001*	-11.1%	-1.93	0.053
Segments	-23.2%	-3.44	<0.001*	-25.7%	-3.57	<0.001*
Edges	-25.5%	-4.87	<0.001*	-19.2%	-3.41	0.002*
Vertices	-25.5%	-4.87	<0.001*	-19.1%	-3.41	0.002*
Sholl AUC	-12.3%	-2.10	0.034*	-25.2%	-4.23	<0.001*

Table 2-2. Summary of alterations in microglial morphology in the entorhinal cortex. % represent percent change from sex-matched VEH controls. * = significant at p<0.05.

	MOR M	MOR F
Fever	↑	↑
Sickness	---	↑
Cytokine Levels (IL-1α)	↑	↑
Microglia Activation	↑	↑
Gut Permeability	---	↑
Anti-LPS Antibody	↓	↓
IgG	↓	↓
IgA	↑	↑
IgM	---	↓

Table 2-3. Summary of results.

3 PERINATAL MORPHINE EXPOSURE INDUCES LONG-TERM CHANGES IN THE GUT MICROBIOTA OF MALE AND FEMALE RATS

3.1 Introduction

Maternal opioid use has risen by 131% since 2010 (Hirai et al., 2021). As a result, a staggering 6.3 per 1000 infants were born in the United States in 2020 experiencing neonatal opioid withdrawal syndrome (NOWS) (Healthcare Cost and Utilization Project, 2022; Hirai et al., 2021). The symptoms associated with NOWS mirror those typically observed in adults and are predominated by gastrointestinal problems, including constipation, nausea, abdominal pain, and diarrhea (Kocherlakota, 2014; Leppert, 2015). Opioid use and misuse has also been linked to gut microbiota dysbiosis, or an altered composition of the commensal bacteria found in the gastrointestinal tract (Akbarali and Dewey, 2019). Both opioid-induced gastrointestinal issues and dysbiosis are a result of opioid action on μ-opioid receptors in the gut, which decrease motility and allow for bacterial overgrowth (Akbarali and Dewey, 2019), predisposing organisms to inflammation, infection, and disease (Round and Mazmanian, 2009; Valdes et al., 2018). Although the microbiota is an essential immune stimulator in early life, very few, if any, clinical studies have investigated gut microbiota composition in infants born with NOWS. It is theorized that exposure to opioids *in utero* leads to a dysbiotic gut microbiota, which is maintained across the lifespan, influencing immune system development (Maguire and Gröer, 2016). Importantly, children exposed to opioids *in utero* have an increased risk of infection (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017), consistent with impaired immune system development.

Chronic opioid exposure in adulthood generally promotes a proinflammatory gut microbiota composition as a result of decreased intestinal transit and increased gut

permeability. Although individual studies report different effects on alpha or beta diversity, it is clear that systemic opioid exposure in adulthood alters gut microbiota composition (Akbarali and Dewey, 2019). Alpha diversity includes richness, or the number of overall microbial species present in a sample, and evenness, or the distribution of abundance of all species in the sample. Previous studies on the influence of opioids on alpha diversity report conflicting results (Lee et al., 2018; Ren and Lotfipour, 2022; Zhang et al., 2021), likely due to differences in the type of opioid administered, the timing and length of dosing schedule, and the time point sampled after opioid exposure; however, the majority report a decrease in alpha diversity with chronic opioid use (Cruz-Lebrón et al., 2021; Gicquelais et al., 2020; Ren and Lotfipour, 2022; Sharma et al., 2020; Wang et al., 2018). Reduced alpha diversity is associated with a variety of negative health outcomes and neurological diseases (Cryan et al., 2020), including autism spectrum disorder, Alzheimer's disease, and epilepsy. Although no clinical studies have investigated alpha diversity in children exposed to opioids *in utero*, this suggests that alpha diversity may be altered long-term, potentially predisposing these children to future health challenges.

Beta diversity, or the differences in the composition of the gut microbiota between groups, is also altered by chronic opioid exposure in adults. Commonly reported changes in gut microbiota composition include expansion of proinflammatory genera of bacteria, including *Staphylococcus, Sutterella, Enterococcus*, and loss of probiotic or protective microbes, including *Lactobacillus*, Lachnospiraceae, and Ruminococcaceae (Fürst et al., 2020). Opioid exposure also reduces the abundance of bile-deconjugating microbes, leading to lower levels of anti-inflammatory bile acids (Banerjee et al., 2016; Wang et al., 2018). Decreases in bile acid levels

are associated with gut barrier disruption and inflammation, further altering gut microbiota composition through decreased secretion of antimicrobial peptides.

The above-mentioned studies focused primarily on chronic opioid use in adults. Thus, there is limited preclinical evidence on the impact of POE on gut microbiota composition. These studies have associated POE with altered alpha (Grecco et al., 2021) and beta diversity (Abu et al., 2021; Grecco et al., 2021; Lyu et al., 2022). Importantly, many of the bacterial taxa identified as differentially abundant after POE are associated with bile acid production (including Ruminococcaceae, Rikenellaceae, Erysipelotrichaceae, Lachnospiraceae, and Allobaculum), suggesting a possible mechanism by which *in utero* opioid exposure influences gut inflammation and barrier function.

Exposure to opioids is associated with a pathogenic profile of the gut microbiota, promoting immune activation, inflammation, and cytokine release. Previous work has associated chronic opioid exposure with increased morbidity and mortality from infection, which was directly related to gut microbiota dysbiosis (Babrowski et al., 2012; Meng et al., 2015; Mora et al., 2011; Wang et al., 2020). These findings are consistent with our previous study in which the febrile and neuroinflammatory response induced by lipopolysaccharide administration is potentiated in perinatally-exposed male and female rats (see Chapter 2). However, the mechanism by which POE alters immune function is unknown. Thus, the present studies were conducted to test the hypothesis that rats perinatally exposed to morphine would have long-term alterations in gut microbiota composition that may contribute to the potentiated response to an immune challenge.

3.2 Methods

3.2.1 Experimental subjects

Female Sprague Dawley rats (approximately two months of age; Charles River Laboratories, Boston, MA) were used to generate offspring. Same-sex pairs or groups of three were co-housed in Optirat GenII individually ventilated cages (Animal Care Systems, Centennial, Colorado, USA) with corncob bedding on a 12:12 hours light/dark cycle (lights on at 8:00 AM). Food (Lab Diet 5001 or Lab Diet 5015 for breeding pairs, St. Louis, MO, USA) and water were provided *ad libitum* throughout the experiment, except during testing. All studies were approved by the Institutional Animal Care and Use Committee at Georgia State University and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to reduce the number of rats used in these studies and minimize pain and suffering.

3.2.2 Perinatal opioid exposure paradigm

Briefly, female Sprague Dawley rats were implanted with iPrecio® SMP-200 microinfusion minipumps at postnatal day 60 (P60) under isoflurane anesthesia. Pumps were programmed to deliver 10-16 mg/kg three times a day. One week after morphine initiation, females were paired with sexually-experienced males for two weeks to induce pregnancy. Morphine exposure to the dams continued throughout gestation. Dams continued to receive morphine after parturition, such that pups received morphine through maternal milk. Beginning at P5, morphine dosage was decreased by 2 mg/kg daily until P7, when morphine administration was discontinued. A separate cohort of rats were implanted with pumps filled with sterile saline to control for the stress of surgery and pump refilling.

3.2.3 Fecal sample collection

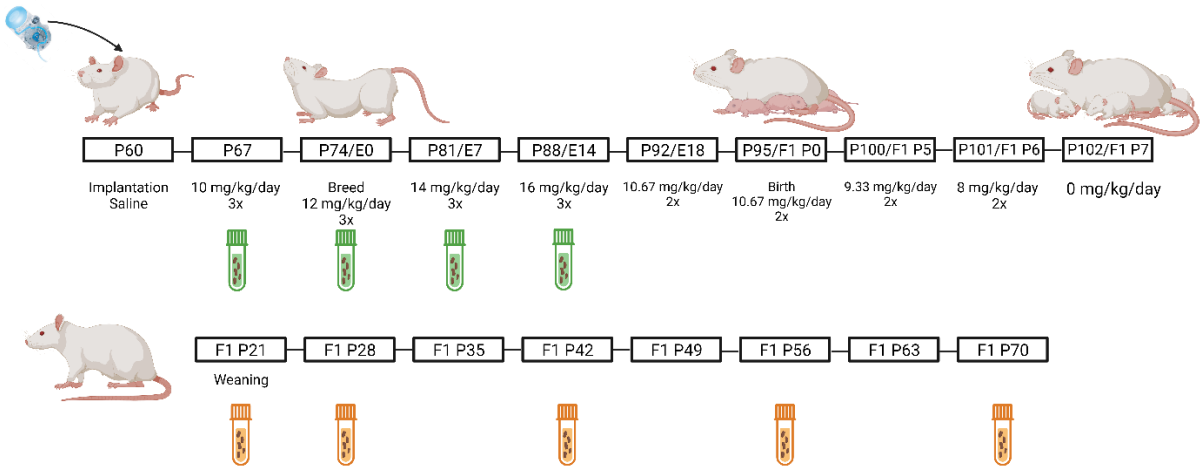


Figure 3-1. Timeline of opioid dosing and sample collection. Green tubes represent fecal sample collection from dams; orange tubes represent collection from offspring.

Fecal samples were collected from both dams and offspring. For dams, rats were isolated into clean containers for fresh fecal collection across the dosing paradigm (10 mg/kg, 12 mg/kg, 14 mg/kg, and 16 mg/kg morphine or the equivalent for vehicle dams). For offspring, morphine-exposed (MOR) and vehicle (VEH) male and female rats were isolated into clean containers for fresh fecal collection at P21, P28, P42, P56, and P70. Due to small sample sizes at P21 and P28, fecal samples were combined per cage at those timepoints (all cages were treatment- and sex-matched). Fecal samples were collected into sterile tubes and promptly frozen at -20°C until sequencing. See Figure 3-1 for a description of the dosing protocol and sampling timepoints.

3.2.4 Microbiota analysis by 16 S rRNA gene sequencing

16S rRNA gene amplification and sequencing were conducted using the Illumina MiSeq technology following the protocol of the Earth Microbiome Project

(https://earthmicrobiome.org/protocols-and-standards/). Bulk DNA was extracted from frozen feces using a QIAamp 96 PowerFecal QIAcube HT kit (Qiagen Laboratories) with mechanical disruption (Qiagen TissueLyser II). The 16S rRNA genes, region V4, were PCR amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which was used to tag PCR products from respective samples (Caporaso et al., 2012; Naimi et al., 2021). The present study used the forward primer 515F 5'-

*AATGATACGGCGACCACCGAGATCTACACGCT*XXXXXXXXXXXX**TATGGTAATT*GT***GTGYCAGCMGCCG CGGTAA-3':

1. the italicized sequence is the 5' Illumina adapter;
2. the 12 X sequence is the Golay barcode;
3. the bold sequence is the primer pad;
4. the italicized and bold sequence is the primer linker;
5. and the underlined sequence is the conserved bacterial primer 515F.

The reverse primer 806R used was 5'-

*CAAGCAGAAGACGGCATACGAGAT***AGTCAGCCAG*CC*** GGACTACNVGGGTWTCTAAT-3':

1. the italicized sequence is the 3' reverse complement sequence of the Illumina adapter;
2. the bold sequence is the primer pad;
3. the italicized and bold sequence is the primer linker;
4. and the underlined sequence is the conserved bacterial primer 806R.

PCR reactions consisted of Hot Master PCR mix (Quantabio, Beverly, MA, USA), 0.2 µM of each primer, and 10–100 ng template; reaction conditions were 3 min at 95°C, followed by 30 cycles of 45 s at 95°C, 60 s at 50℃, and 90 s at 72°C on a Biorad thermocycler. Products were then visualized by gel electrophoresis and quantified using Quant-iT PicoGreen dsDNA assay (Clariostar Fluorescence Spectrophotometer). A master DNA pool was generated in equimolar ratios, subsequently purified with Ampure magnetic purification beads (Agencourt, Brea, CA,

USA) and sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 × 250 bp) at the Genom'IC platform (INSERM U1016, Paris, France).

3.2.5 16S rRNA gene sequence analysis

16S rRNA sequences were analyzed using QIIME2—version 2019 as previously described (Naimi et al., 2021). Sequences were demultiplexed and quality filtered using the Dada2 method with QIIME2 default parameters in order to detect and correct Illumina amplicon sequence data, and a table of QIIME2 artifacts was generated. A tree was next generated, using the align-to-tree- mafft-fasttree command, for phylogenetic diversity analyses; alpha and beta diversity analyses were computed using the core-metrics-phylogenetic command. To compare species richness and evenness, three measures of alpha diversity were calculated: Pielou's evenness, Shannon's index, and Simpson's index. Principal coordinate analysis (PCoA) plots were used to assess the variation between the experimental groups (beta diversity) as generated with the R package qiime2R v0.99.6, along with PERMANOVA for statistical analysis of treatment differences (as implemented in Qiime2 using the beta-group-significance command). Two measures of beta diversity were calculated: Bray-Curtis dissimilarity and unweighted Unifrac distance. For taxonomy analysis, features were assigned to operational taxonomic units (OTUs) with a 99% threshold of pairwise identity to the Greengenes reference database 13_8.

3.2.6 Microbiota analysis using Maaslin2 to identify differentially abundant OTUs

To determine which OTUs were differentially expressed across metadata categories (i.e., drug treatment, sex, and/or age), we utilized the R package MaAsLin2 v1.10.0 (Mallick et al., 2021). MaAsLin2 utilizes general linear models, metadata, and rarefied taxonomic data to

compare abundance of individual OTUs between metadata categories. Abundance was compared both for MOR vs. VEH dams across the dosing schedule (10 mg/kg, 12 mg/kg, 14 mg/kg, 16 mg/kg morphine or equivalent for saline). We also compared MOR vs. VEH offspring across age (P21, P28, P42, P56, and P70) separately by sex. For offspring, random effects of litter and experimental cohort were also included. For analysis, min_prevalence was set to 0.1 (the minimum percentage of samples an OTU must be detected at minimum abundance for further analysis; 10%) and min_abundance was set to 0.0001 (the minimum abundance for each OTU; non-zero). All other parameters used default values. Individual OTUs were compared between treatment, sex, and age to generate coefficients of the individual comparisons, along with p values to represent significance. Those p values were then corrected for multiple comparisons via the Benjamini-Hochberg false discovery rate correction to generate q values. Any differences with $q<0.05$ were considered significant. Effect sizes were calculated via -log(qvalue), multiplied by the sign of the coefficient of the difference between treatment groups. Positive effect sizes represent higher abundance in MOR rats. All comparisons were made to the sex- and age-matched vehicle controls.

3.2.7 Microbiota analysis using maturity-index in Qiime2

As we examined gut microbiota composition in MOR and VEH rats across multiple ages (P21, P28, P42, P56, and P70), we also investigated “microbial maturity” using the maturity-index function in Qiime2. By comparing gut microbiota composition over time in a reference group (in this case, VEH rats), maturity-index generates a regression model to predict age as a function of composition and then calculates maturity index z-scores (MAZ scores) to compare relative microbial maturity across groups. Lower MAZ scores represent relatively “younger”

microbial maturity. We also utilized the MAZ scores in a linear mixed-effects model, as implemented in Qiime2 with the longitudinal linear-mixed-effects function, in order to determine whether treatment or sex was a significant predictor of maturity z-score.

3.2.8 Experimental design and statistical analysis

Female rats were randomly assigned to the morphine or vehicle condition. All experiments included both male and female offspring to investigate potential sex-specific effects of perinatal opioid exposure on gut microbiota composition. No differences were observed between rats of different litters in the same drug exposure group (i.e., MOR and VEH); therefore, individual rats from multiple litters served as a single sample count (3 VEH litters, 3 MOR litters). Data was collected from two separate rounds of rats. All analyses were completed blinded to treatment group.

Alpha diversity was analyzed using mixed models with Greenhouse-Geisser correction as implemented in GraphPad Prism 9.1.0 (Motulsky, 2023) to identify significant effects of treatment and dose/age; $p<0.05$ was considered significant. Sidak's post-hoc tests were conducted to determine significant mean differences between *a priori* specified groups.

3.3 Results

3.3.1 Maternal alpha and beta diversity

As maternal microbiota would be transferred to the offspring at birth and form the basis for the offspring's gut microbiota, we first investigated the direct effects of morphine exposure on female rats before pregnancy and across gestation. Previous studies suggest that alterations in maternal microbiota during gestation can lead to long-term changes in offspring's gut microbiota composition and immunity (Nyangahu et al., 2018), suggesting that maternal microbiota dysbiosis has a potential negative influence on offspring's future health. Three specific measures of alpha diversity were analyzed at each morphine dose (10, 12, 14, and 16 mg/kg) and compared to time-matched vehicle samples: Pielou's evenness (Figure 3-2A), Shannon's index (Figure 3-2B), and Simpson's index (Figure 3-2C). No significant effects of treatment or dose were found in any of the three alpha diversity metrics (see Table 3-1). Although not significant, alpha diversity generally decreased across pregnancy in both groups, suggesting that the effects of pregnancy may overshadow any effects of morphine.

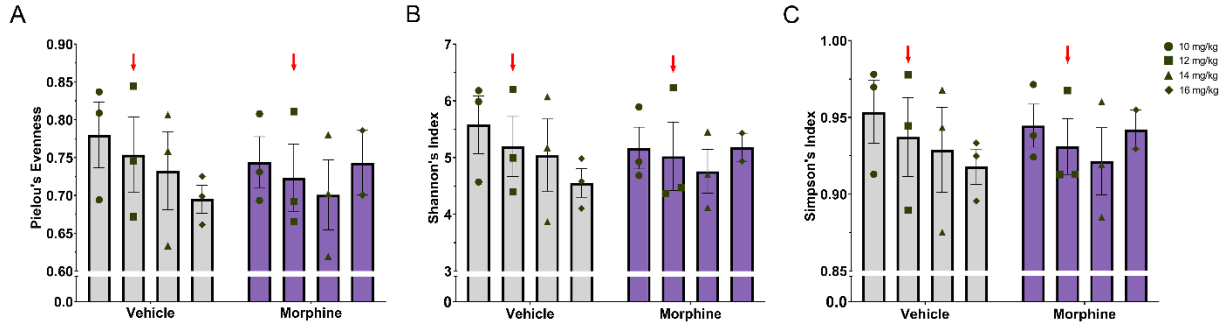


Figure 3-2. Alpha diversity does not differ between morphine- and vehicle-treated dams. **A.** No differences in Pielou's evenness (**A**), Shannon's index (**B**), or Simpson's index (**C**). Red arrows indicate the initiation of breeding. $N_{VEH} = 3$, $N_{MOR} = 3$.

We next investigated beta diversity to compare gut microbiota composition between VEH and MOR dams. Two different measures of beta diversity were utilized: Bray-Curtis dissimilarity and unweighted Unifrac distances. Bray-Curtis is a non-phylogenetic and weighted measure, and unweighted Unifrac is a phylogenetic and unweighted measure. PERMANOVA was used to determine if beta diversity significantly differed between VEH and MOR dams. Significant treatment effects were observed in both Bray-Curtis dissimilarity (p=0.019; Figure 3-3A) and unweighted Unifrac distances (p=0.042; Figure 3-3B), suggesting altered gut microbiota composition as a result of morphine treatment.

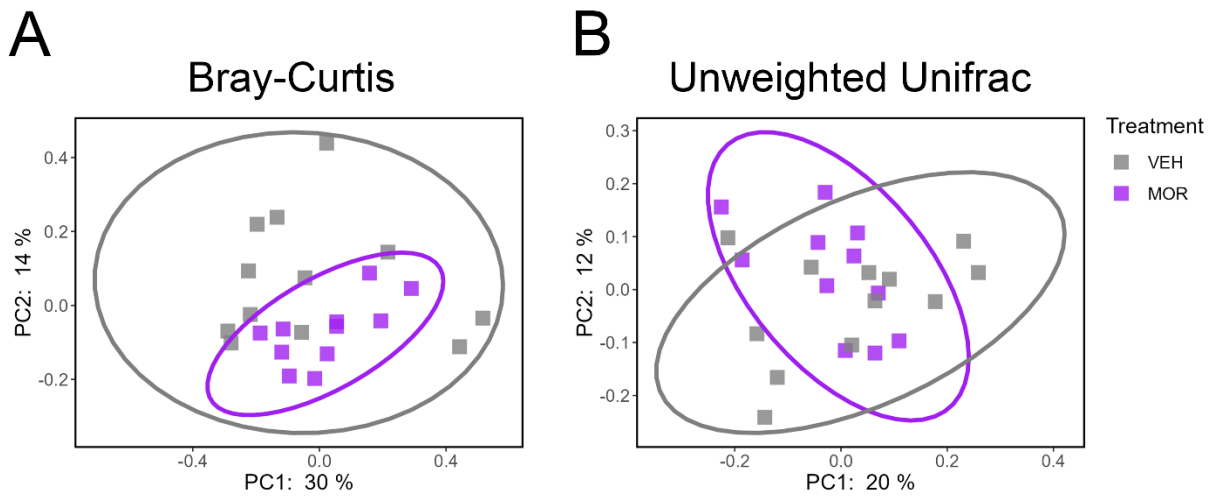


Figure 3-3. Morphine-exposed dams have significantly altered beta diversity in comparison to vehicle dams. **A.** Significant clustering on the Bray-Curtis dissimilarity measure based on treatment. **B.** Significant clustering of unweighted Unifrac distances based on treatment. N_{VEH} = 3, N_{MOR} = 3.

We next examined which OTUs were responsible for the alteration in beta diversity. For this, we utilized MaAsLin2 to compare abundance between VEH and MOR dams at all four timepoints/morphine doses investigated. At 10 mg/kg, when morphine was first initiated and prior to breeding, two OTUs were significantly increased in MOR dams: an unidentified Ruminococcaceae member and the genus *Oscillospira*. Both the Ruminococcaceae family and

its genus *Oscillospira* are generally considered probiotic and involved in both bile acid and short-chain fatty acid synthesis (Fürst et al., 2020; Yang et al., 2021). No other OTUs were significantly altered at any other time, suggesting that opioids may have larger effects at initiation vs. maintenance.

3.3.2 Offspring alpha and beta diversity

We next investigated alpha and beta diversity of male and female VEH and MOR offspring. There was no significant effect of sex in any of the three alpha diversity metrics so data were collapsed by sex to increase power. No significant effects of age or treatment were noted in Pielou's evenness (Figure 3-4A; p=0.1198) or Simpson's index (Figure 3-4C; p=0.2744); see Table 3-1 for complete statistics. However, Shannon's index (Figure 3-4B) identified a significant treatment*age interaction (p=0.0178). At P21 and P28, MOR rats had increased Shannon's index, followed by decreased Shannon's index at P42 and P56, and no differences at P70. However, given the small magnitude of change and the resolution of any differences by P70, perinatal opioid exposure seems to have minor effects of alpha diversity.

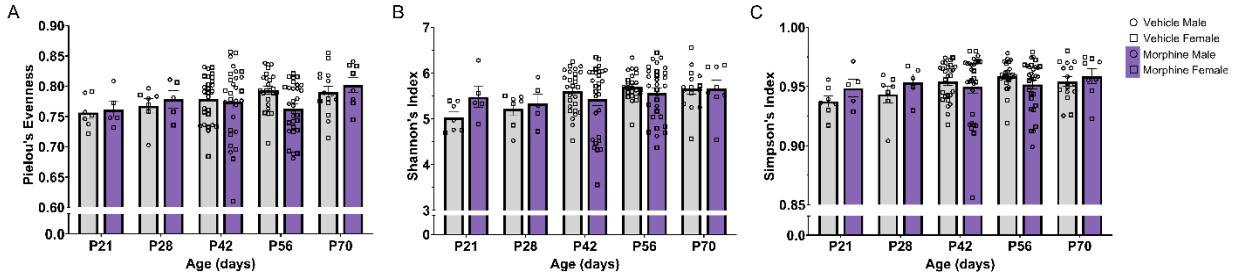


Figure 3-4. Minor differences were observed in alpha diversity of morphine-exposed offspring. **A.** No significant differences in Pielou's evenness. **B.** Significant treatment*age interaction in Shannon's index. **C.** No significant differences in Simpson's index. N_{VEH} = 7-27, N_{MOR} = 5-30.

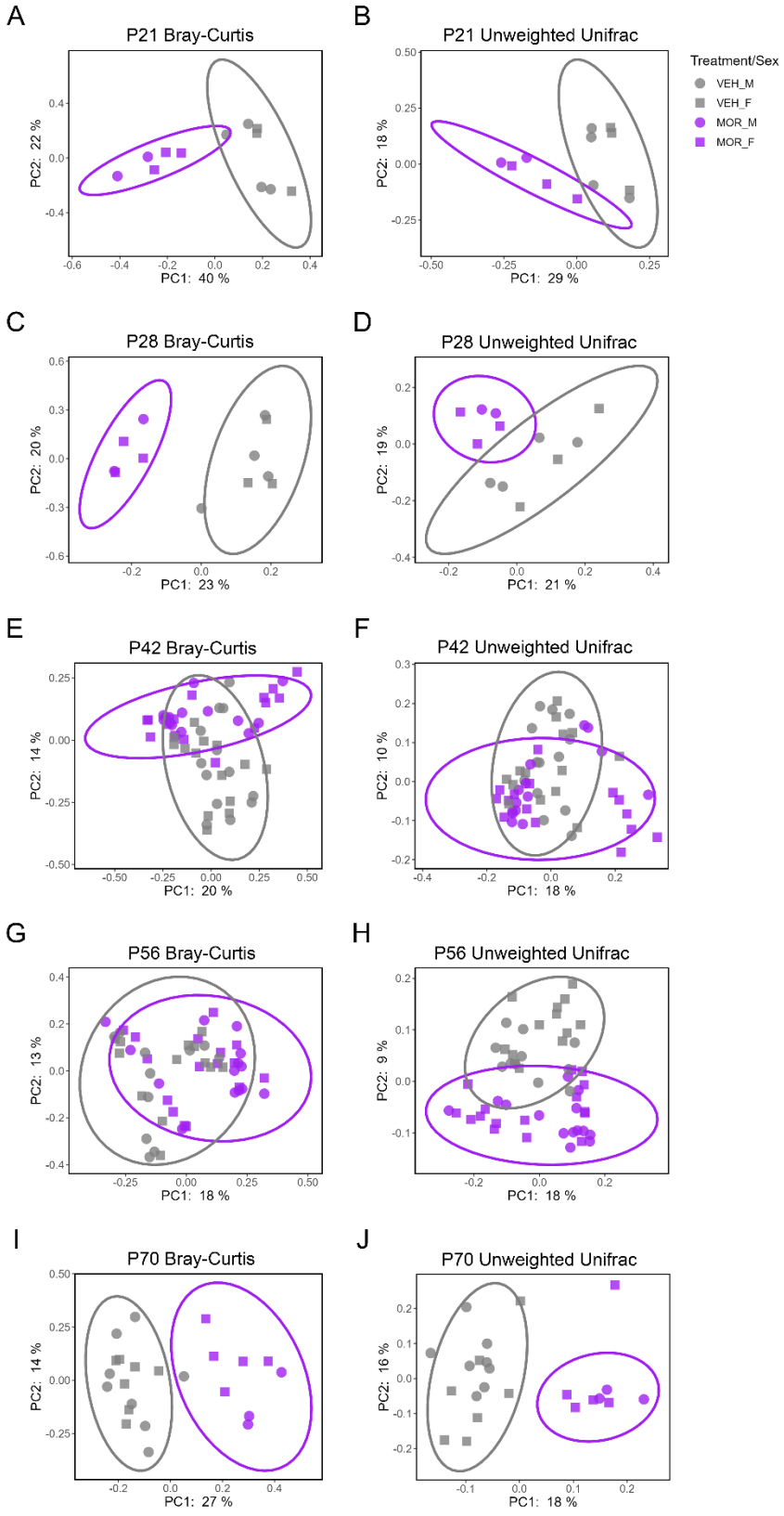


Figure 3-5. Significant effects of treatment on Bray-Curtis dissimilarity (**A**, P21; **C**, P28; **E**, P42; **G**, P56; **I**, P70) and unweighted Unifrac distances (**B**, P21; **D**, P28; **F**, P42; **H**, P56; **J**, P70).

We next analyzed beta diversity separately at P21, P28, P42, P56, and P70 using both Bray-Curtis and unweighted Unifrac (Figure 3-5). At P21, both Bray-Curtis (p=0.01; Figure 3-5A) and unweighted Unifrac (p=0.023; Figure 3-5B) identified significant clustering based on treatment and sex, although no individual comparisons of treatment/sex groups reached statistical significance. At P28, neither Bray-Curtis (p=0.071; figure 3-5C) nor unweighted Unifrac (p=0.2; Figure 3-5D) identified significant differences in beta diversity; however, when sex was collapsed, there was a significant effect of treatment for both metrics (p=0.001 and p=0.003, respectively). At P42 (Bray-Curtis, p=0.001, M: q=0.0165, F: q=0.0160; unweighted Unifrac, p=0.001, M: q=0.0015; F: q=0.0015), P56 (Bray-Curtis, p=0.001, M: q=0.0015, F: q=0.0015; unweighted Unifrac, p=0.001, M: q=0.004, F: q=0.003), and P70 (Bray-Curtis, p=0.001, M: q=0.0075, F: q=0.006; unweighted Unifrac, p=0.001, M: q=0.024, F: q=0.03), both Bray-Curtis and unweighted Unifrac identified significant differences in beta diversity for both males and females (Figures 3-5E-J). In summary, across all five ages sampled, beta diversity was significantly different between VEH and MOR rats, with effects of sex at some ages. Interestingly, although MOR rats were only exposed indirectly to opioids until P7, the observed changes in composition were maintained at least until P70 and even expanded with age.

We next analyzed the abundance of specific OTUs responsible for the observed differences in beta diversity noted across age.

At P21 (Figure 3-6A-B), 19 features were differentially abundant (M = 10, F = 6, both = 3), and all 19 were elevated in MOR rats. Of the differentially abundant features, 63.6% were members of the Clostridiales order, particularly the Lachnospiraceae family. Mixed evidence has been reported on the effects of Lachnospiraceae on host health (Vacca et al., 2020), but

many Lachnospiraceae members are involved in short-chain fatty acid synthesis, which is important in health and disease.

At P28 (Figures 3-6C-D), 11 features were differentially abundant (M = 5, F = 4, both = 2), with seven upregulated in MOR rats. The families Lachnospiraceae and S24.7 accounted for over half of the differentially abundant features. Although little is known about the role of S24.7, it is a common member of both the rodent and human gut microbiota (Ormerod et al., 2016) and occupies a unique functional niche in the gut, likely playing a role in health and disease.

At P42 (Figures 3-6E-F), ten features were differentially abundant (M = 3, F = 6, both = 1), all downregulated in MOR rats; these included members of the Lachnospiraceae and S24.7 families. Similar results were observed at P56 (Figures 3-6G-H). Ten features were differentially abundant (M = 6, F = 4), eight of which were downregulated in MOR rats. Many differentially abundant OTUs were members of the Lachnospiraceae and Ruminococceae families. At P70 (Figures 3-6I-J), 17 features were differentially abundant (M = 11, F = 5, both = 1), 12 of which are upregulated. More than 75% of those differentially abundant OTUs were members of the Clostridiales order, including Lachnospiraceae and Ruminococcaceae.

Together, this suggests that perinatal exposure to morphine leads to long-term alterations in gut microbiota composition, particularly the Clostridiales order (primarily the Lachnospiraceae and Ruminococcaceae families) and the S24.7 family, both of which were altered at all five timepoints.

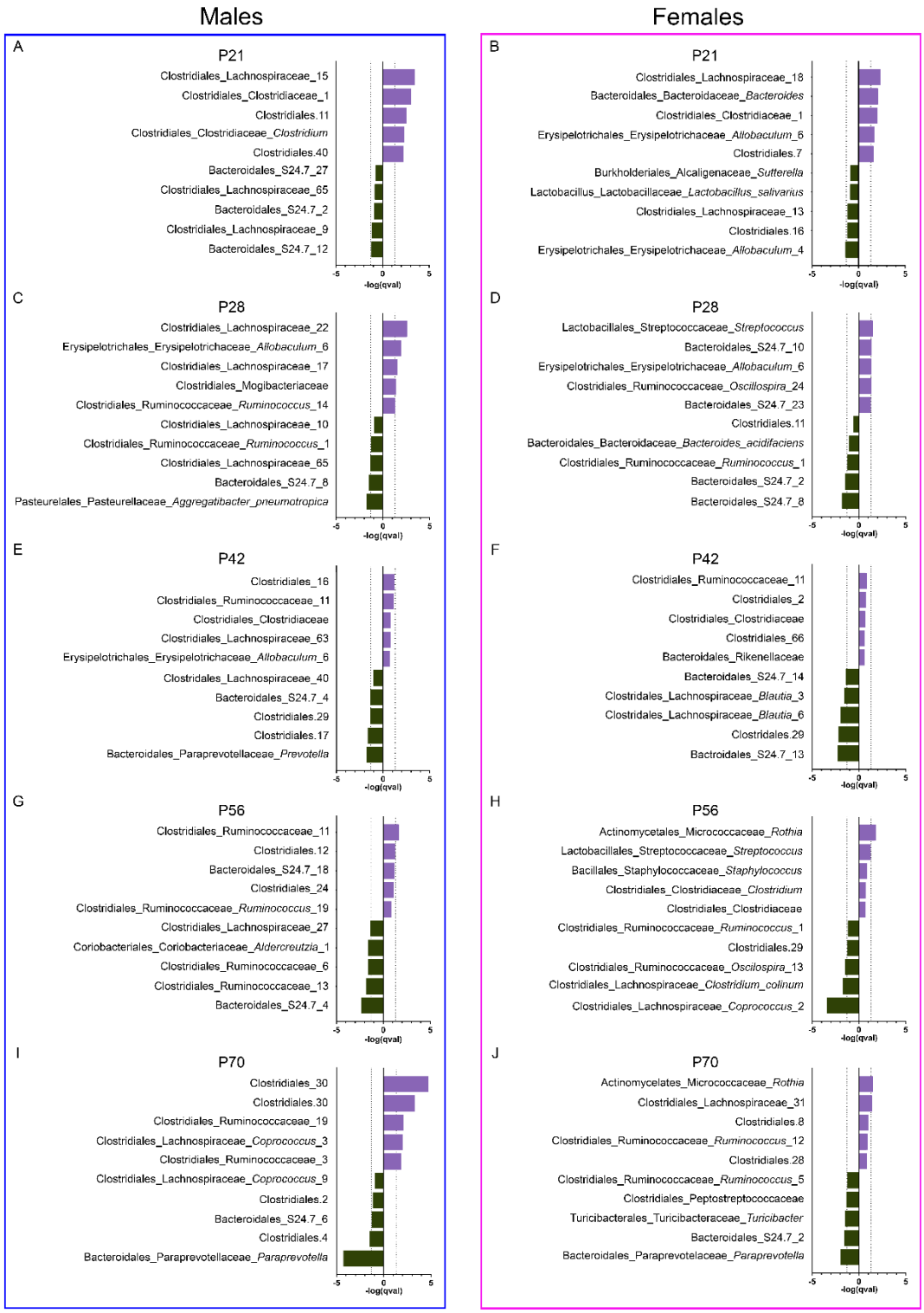


Figure 3-6. Top five significantly increased and decreased OTUs in the microbiota of morphine-exposed male (**A**, **C**, **E**, **G**, **I**) and female (**B**, **D**, **F**, **H**, **J**) rats at P21 (**A-B**), P28 (**C-D**), P42 (**E-F**), P56 (**G-H**), and P70 (**I-J**). Purple bars represent OTUs increased in the morphine group; gray bars represent OTUs decreased in the morphine group. Dotted vertical lines represent the threshold for significant effect sizes. OTU names = Order_Family_Genera_Species.

3.3.3 Analysis of microbial maturity

As our analysis included multiple timepoints, we next investigated microbial maturity using the maturity-index function, which trains a regression model on a subset of VEH composition data to compare composition changes as rats age. Maturity index z-scores (MAZ scores) are then calculated to compare the relative maturity between VEH and MOR rats. Negative MAZ scores indicate relatively "younger" gut microbiota composition, suggesting a distinct microbial state. MAZ scores were higher for MOR rats at P21 and P28, with average z-scores of 2.74 and 0.71, respectively. However, at P42, P56, and P70, MAZ scores were considerably lower, with average z-scores of -0.69, -1.57, and -1.73, respectively (Figure 3-7A). This suggests that as MOR rats age, their gut microbiota composition does not mature in a comparable manner to VEH rats. We also generated a predicted age based on microbiota composition and compared that to the actual age of the rat at sampling. Positive values indicate a relatively "older" gut microbiota composition than the actual age at sampling; negative values indicate relatively "younger" samples. At both P21 and P28, MOR microbiota composition appeared more mature, with an average predicted age of 5.45 and 6.66 days older than the actual sampling age. However, at P42, P56, and P70, MOR microbiota composition was less mature, with an average predicted age difference of 2.86, 6.27, and 9.38 days younger than the actual sampling age (Figure 3-7B). This suggests that as MOR rats reach adulthood, their gut microbiota composition remains less mature.

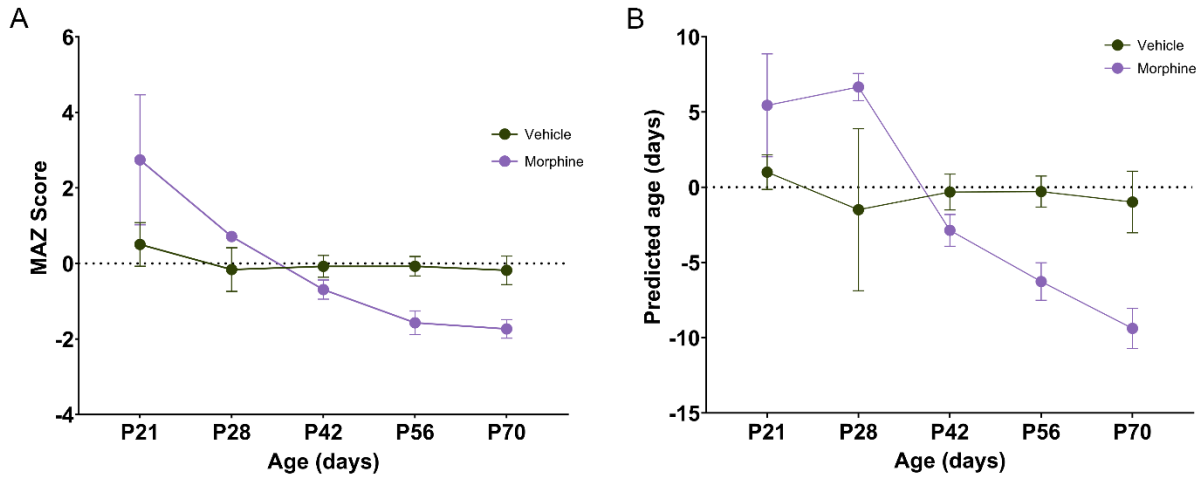


Figure 3-7. Microbial maturity is altered in morphine-exposed offspring. **A.** MAZ scores for morphine-exposed rats are higher at P21 and P28 but lower at P42, P56, and P70. **B.** Predicted microbial age is higher for morphine-exposed rats at P21 and P28 but lower at P42, P56, and P70. N_{VEH} = 3-15, N_{MOR} = 5-30.

We next utilized a linear mixed effects model to determine if treatment, sex, or age were significant predictors of maturity index z-scores. As expected, age was a significant predictor of maturity index z-scores ($p<0.001$) along with treatment ($p=0.01$). However, sex was not a significant predictor ($p=0.608$).

3.4 Discussion

The present study was designed to investigate the potential impact of perinatal morphine exposure on gut microbiota composition. We hypothesized that as a result of vertical transmission of a dysbiotic microbiota along with the direct action of opioids on the perinatal gut, the gut microbiota composition of morphine-exposed offspring would be dysbiotic, potentially contributing to the immune dysregulation previously reported in both clinical and preclinical studies of POE (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017) (see Chapter 2). Here, we report that morphine administration to dams across pregnancy had a minimal

effect on gut microbiota composition; in contrast, long-term and robust changes in gut microbiota composition were observed in male and female MOR offspring.

In our initial studies, we investigated gut microbiota composition in morphine- and vehicle-treated dams, as the maternal microbiota has a major influence on the microbiota composition of the offspring. Unexpectedly, no significant differences in alpha diversity were noted in dams treated with morphine across gestation. Rather, alpha diversity generally decreased in both vehicle- and morphine-treated dams as a function of time pregnant, suggesting that pregnancy may have a greater impact on microbiota composition than morphine treatment, at least at the doses tested in this study. Gut microbiota composition is dramatically altered across pregnancy: at initiation, composition remains similar to the non-pregnant state, but as pregnancy progresses, the abundance of proinflammatory OTUs increases (Edwards et al., 2017). These physiological changes in the gut microbiota are theorized to alter maternal metabolism in order to support and maintain the pregnancy; however, although not observed here, excessive proinflammatory microbial expansion is associated with a variety of negative outcomes for both the mother and fetus (Edwards et al., 2017). Significant differences were noted in beta diversity in both Bray-Curtis dissimilarity and unweighted Unifrac distance; however, only two OTUs were differentially abundant between vehicle- and morphine-treated dams. Both significant differences were observed before breeding (10 mg/kg), suggesting that morphine's largest impact on the microbiota is at initiation, and not maintenance.

Despite relatively minor differences observed when comparing vehicle- and morphine-exposed dams, morphine-exposed offspring had significant changes in beta diversity, which

were maintained into adulthood and thus, likely permanent. For alpha diversity, although no differences were observed in Pielou's evenness or Simpson's index, there were minor (albeit significant) differences in Shannon's index, although these changes were mainly resolved by P70. Despite only two OTUs showing significant changes in morphine-exposed dams, robust and prolonged differences were observed in the offspring. Together, this suggests that although maternal microbiota composition is one driver of offspring composition, morphine can also act directly on the perinatal gut to alter permeability and peristalsis, creating a conducive environment for microbes to flourish. Interestingly, these changes were maintained into adulthood (P70), despite opioid exposure ending at P7, suggesting that morphine induced long-term damage to the gut and microbiota.

The OTUs differentially expressed in the morphine-exposed offspring over all five ages examined generally belonged to the families Lachnospiraceae, Ruminococcaceae, and S24-7. Although studies have implicated these families in both a health- and disease-promoting role, alterations in such highly abundant members of the microbiota suggest that these changes provide an underlying mechanism whereby infants exposed to opioids *in utero* show a compromised immune response, resulting in higher rates of infection and hospitalization (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017). Previous studies in adults utilizing chronic opioid exposure report that transplantation of morphine-associated microbiota recapitulates opioid-induced gut pathologies and alterations in immunity (Banerjee et al., 2016). Future studies on perinatal opioid exposure should consider the causal role of gut microbiota composition on opioid-associated immune function by utilizing fecal transplant studies, which

may eventually facilitate the development of treatment strategies for children exposed to opioids *in utero*.

Although the mechanism by which opioid-exposed microbiota influences immune system development is unknown, the families Lachnospiraceae and Ruminococcaceae are implicated in bile acid and short chain fatty acid production (Murakami et al., 2018; Vacca et al., 2020), both of which are essential modulators of gut health, microbiota composition, and inflammation. As the majority of OTUs dysregulated in the current study were members of the Lachnospiraceae and Ruminococcaceae families, this suggests that perinatal opioid exposure directly influences bile acid and short chain fatty acid production, paralleling previous work examining chronic opioid exposure in adults (Banerjee et al., 2016; Cruz-Lebrón et al., 2021).

Our results demonstrating a profound and likely permanent impact of perinatal morphine on gut microbiota composition are poised to have a significant impact on the field. Importantly, this study is the first to use a clinically-relevant administration protocol that recapitulates the opioid use profile of pregnant women, including both prenatal and gestational opioids, pulsatile dosing, and increased dosing through pregnancy to account for tolerance. In addition, although previous studies generally report changes in Lachnospiraceae (Grecco et al., 2021), *Lactobacillus*, *Ruminococcus*, *Allobaculum* (Abu et al., 2021), and Clostridia (Lyu et al., 2022), all of which were also altered in the current study, those studies are limited in scope, typically sampling offspring at one age (Grecco et al., 2021; Lyu et al., 2022) or timepoints limited to weaning (Abu et al., 2021) when the gut microbiota composition is still in flux as a result of diet change. Our studies are the first to report that opioid-induced gut microbiota dysbiosis is not only present during early life, but is maintained into adulthood.

In the current study, we also report that morphine-exposed male and female rats had significantly lower microbial maturity in adulthood. Lower microbial maturity is generally defined as a distinct microbial state, rather than a lack of maturity (Subramanian et al., 2014), meaning that MOR rats have distinct composition and developmental trajectory. Lower maturity has been reported in severely malnourished children and was maintained over months despite the introduction of therapeutic food (Subramanian et al., 2014). This suggests that early perturbations of gut microbiota development are maintained long-term. Furthermore, children born premature and admitted to the neonatal intensive care unit had lower microbial maturity vs. healthy controls, and lower microbial maturity was associated with higher beta diversity volatility in early childhood (Yee et al., 2019). Beta diversity volatility, or the change in beta diversity over time, is considered a hallmark sign of disease, as the microbiota should generally remain stable after weaning and solid food introduction (Bokulich et al., 2018). Together, this suggests that morphine-exposed male and female rats have lower microbial maturity, and that this lower maturity may be associated with an increased disease risk and beta diversity volatility.

The results of this study have significant clinical implications for children born with NOWS, who often suffer from gastrointestinal complications and are at higher risk for infection later in life. Though it is currently theorized that *in utero* opioid exposure alters gut microbiota composition in humans (Maguire and Gröer, 2016), to date, no clinical studies have compared the microbiota of children exposed to opioids *in utero* to healthy controls. As a consequence, the mechanism by which opioid-exposed microbiota influences human health or increases disease risk later in life is currently unknown.

In summary, the current study provides evidence that perinatal morphine leads to long-term changes in gut microbiota composition in male and female offspring, potentially having a direct impact on the immune system later in life (see Chapter 2). Specifically, we report here that members of the families Lachnospiraceae, Ruminococcaceae, and S24-7 are altered until at least P70 and that the maturity of the morphine-exposed microbiota composition is lower than that of vehicle rats in adulthood. The use of a clinically translatable model of *in utero* opioid exposure provides initial evidence to support the hypothesis that human infants born with NOWS suffer from gut dysbiosis, potentially leading to long-term negative health outcomes and increased risk for infection and hospitalization.

	Alpha Diversity Metric	Treatment	Dose/Age	Interaction
Dam	Pielou's Evenness	F(1,4)=0.05242, p=0.8285	F(2.350,8.618)=1.223, p=0.3484	F(3,11)=1.135, p=0.3776
	Shannon's Index	F(1,4)=0.01303, p=0.9146	F(1.964,7.202)=0.8796, p=0.4534	F(3,11)=0.7716, p=0.5337
	Simpson's Index	F(1,4)=0.0007879, p=0.9790	F(2.123,7.786)=1.546, p=0.2733	F(3,11)=0.8077, p=0.5155
Offspring	Pielou's Evenness	F(1,55)=0.2744, p=0.6025	F(3.158,72.61)=1.991, p=0.1198	F(4,92)=2.259, p=0.0687
	Shannon's Index	F(1,55)=3.264, p=0.0763	F(3.086,70.97)=2.451, p=0.0688	F(4,92)=3.152, p=0.0178*
	Simpson's Index	F(1,55)=2.470, p=0.1218	F(3.387,77.90)=1.366, p=0.2573	F(4,92)=2.263, p=0.0683

Table 3-1. F values for alpha diversity metrics for dams and offspring. * = significant at $p<0.05$.

4 DISCUSSION

4.1 Summary: Perinatal exposure to morphine leads to long-term alterations in immune functioning and gut microbiota composition

6.3 per 1000 hospital births were diagnosed with neonatal opioid withdrawal syndrome (NOWS) in 2020 (Healthcare Cost and Utilization Project, 2022). The escalation in rates is directly related to the rise in opioid use and abuse among women of reproductive age (Hirai et al., 2021), including a 131% increase in maternal opioid use since 2010. Although little clinical evidence is available documenting the long-term consequences of perinatal opioid exposure (POE), retrospective chart reviews have identified an increased risk of hospitalization for infection later in life (Arter et al., 2021; Uebel et al., 2015; Witt et al., 2017). This data is consistent with preclinical and clinical studies reporting that chronic opioid consumption induces immunosuppression and gut microbiota dysbiosis in adult humans and rodents (Vallejo et al., 2004).

Although the increased infection risk observed in children exposed to opioids in utero has been attributed to lifestyle issues or poor parenting (Oei, 2018), the elevated rate is maintained even after controlling for other drug exposures, poor home environment, and prematurity. Therefore, it is likely that POE has a direct effect on the developing fetus that leads to long-term alterations in immune system functioning. One hypothesis is that POE leads to gut microbiota dysbiosis, or an altered composition of the commensal microbes in the gastrointestinal tract (Maguire and Gröer, 2016). Dysbiosis during critical periods of development has been previously linked to alterations in immune system maturation (Maguire and Gröer, 2016), providing a possible mechanism by which *in utero* opioid exposure leads to

long-term changes in immune reactivity. Given the known effects of opioids on gut microbiota dysbiosis and immune function in adulthood, and the essential role the gut microbiota plays in shaping the development of the immune system, we hypothesized that POE would lead to long-term gut microbiota dysbiosis and altered immune response.

The data presented in Chapters 2 and 3 demonstrate that perinatal opioid exposure affects both baseline immunity as well as the response to the immune stimulator lipopolysaccharide (LPS). More specifically, morphine-exposed (MOR) rats had increased gut permeability, particularly females, but had lower levels of anti-LPS antibodies and the antibody classes IgG and IgM. This indicates that although the immune system is being challenged with more microbial contact via the leaky gut, it is unable to properly mount an antibody response. Furthermore, IgA levels were dramatically increased in MOR rats, likely as a function of IgA's role in mucosal immunity, gut barrier function, and as an antimicrobial. When challenged with LPS, MOR rats had increased fever and sickness, cytokine levels, and activation-associated microglia morphology. These results parallel the similar peripheral immune deficits observed in adult rats treated with opioids. Furthermore, we demonstrate that despite morphine exposure occurring from E0-P7, profound gut microbiota dysbiosis was induced and maintained until at least P70. As the microbiota is an essential training stimulus for proper immune system development, this data suggests that gut microbiota dysbiosis as a result of POE contributed to the deficits we observed in immune function.

4.2 Perinatal exposure to morphine alters baseline immune function and the response to lipopolysaccharide

The present studies investigate immune function both at baseline (gut permeability and antibody production) and following induction of a model of Gram-negative bacterial infection (lipopolysaccharide; LPS). LPS is the most commonly used experimental immune activator, and has been used extensively as a readout of immune function, including in our laboratory (Doyle et al., 2017). Although some studies utilize direct bacterial infections to investigate immunity, LPS is preferred for its more uniform and predictable response (Zeisberger, 1999). Importantly, the LPS-induced fever is evident within 3-4 hours post-administration and is an antibody-mediated response. This is in contrast to the second phase of the immune response (including T cell proliferation) that occurs later (>8 hours after exposure). Following exposure, peripheral immune cells with immunological memory recognize LPS as foreign and initiate cytokine production. These cytokines are then transported through the circulatory system to the median preoptic area (MnPO) of the hypothalamus, where they (primarily IL-1) initiate prostaglandin E2 synthesis to increase core body temperature through brown adipose tissue metabolism and vasoconstriction (Hart, 1988; Machado et al., 2020; Saper and Breder, 1994; Zampronio et al., 2015).

In the present studies, rats exposed to opioids *in utero*, particularly females, displayed an elevated fever and sickness response to LPS. These rats also had increased cytokine levels and activation-associated microglial morphology within the periaqueductal gray (PAG) post-LPS administration. At baseline, perinatal opioid exposure was associated with increased gut permeability and decreased antibody levels, indicating possible immunosuppression that

altered the response to LPS. Morphine-exposed rats also displayed robust and prolonged gut microbiota dysbiosis.

4.3 Results support long-term leukopenia in rats perinatally exposed to morphine

Opioids have previously been shown to induce leukopenia, or a low count of leukocytes in the blood. Neutropenia (low count of neutrophils) and lymphopenia (low count of lymphocytes), both subclasses of leukocytes, have also been reported. Interestingly, rats made leukopenic due to chemotherapy display increased LPS-induced fever similar to what we observed (Miñano et al., 2004; Tavares et al., 2006, 2005). This suggests that reduced leukocyte counts may have contributed to the increased febrile response to LPS in MOR rats. Opioid treatment in adult rats induces leukopenia through a decrease in lymphocyte proliferation (Flores et al., 1995; Hamra and Yaksh, 1996). Lymphocyte proliferation is an essential first step in the cell-mediated immune response to generate effector or memory lymphocytes in response to mitogens or antigens. Thus, an improper initial proliferation response has a cascading negative effect on the rest of the adaptive immune response. When treated with LPS, rats made severely leukopenic by chemotherapy treatment display robust and potentiated fever (Miñano et al., 2004; Tavares et al., 2006, 2005). Although it initially seems contradictory for immunosuppression to produce an increased response to immune challenge, it is proposed that the loss of antipyretic molecule production from leukocytes and release of pyrogens from resident Kupffer macrophages in the liver promotes higher fever (Miñano et al., 2004; Tavares et al., 2006, 2005). In rats with normal leukocyte levels, production of antipyretic cytokines such as IL-10 would balance the generation of pyrogens such as IL-1 and IL-6 to avoid excessive fever generation (Tavares et al., 2006). Therefore, a decrease in lymphocytes as a result of

perinatal opioid exposure may be one potential explanation for the potentiated febrile response to LPS observed in the current study. Furthermore, clinical evidence suggests that children born exposed to opioids *in utero* have an increased rate of and/or risk factors for leukopenia (Adatia et al., 2021; Culver et al., 1987; Miller et al., 2022; Romanos-Sirakis et al., 2020). Therefore, we propose the following model: perinatal opioid exposure induces leukopenia as a result of decreased lymphocyte proliferation; when treated with LPS, fewer antipyretic molecules are produced as a result of the lower cell count, which allows fever to increase dramatically (see Figure 4-1).

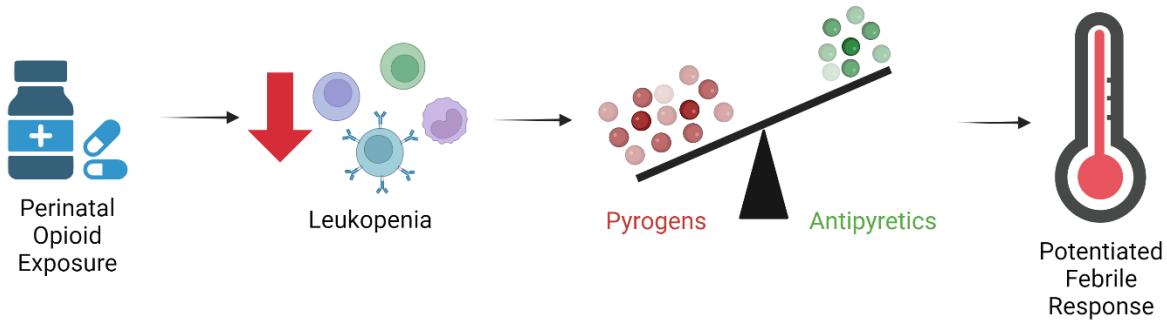


Figure 4-1. Perinatal opioid exposure induces leukopenia, which causes a shift in the balance of pyrogens and antipyretics. This shift induces higher fever in morphine-exposed rats. Created with Biorender.

4.4 Increased sickness-associated features and cytokine response as a result of perinatal opioid exposure

One major aspect of the innate immune system associated with fever is sickness behavior, which includes many features, including fatigue, reduced appetite and food consumption, and social withdrawal (Lasselin, 2021) and is theorized to conserve energy and divert resources to the immune system. In rodents as well as humans, facial cues are an effective metric of sickness (Sotocinal et al., 2011). LPS induces robust sickness-associated

features, including ears laying back against the head, nose/cheek flattening, squinted eyes, and piloerect fur. By scoring each of the four sickness-associated features from 0 (not present) to 2 (present and severe), we are able to observe and compare sickness between VEH and MOR rats. Importantly, sickness scores correlate with LPS-induced fever (see Chapter 2). Sickness was increased in morphine-exposed female rats, consistent with the potentiated febrile response observed in MOR rats. Even though MOR and VEH male rats scored very high on the sickness scale, VEH female rats, despite a robust febrile response, showed very few signs of being sick. Male rats typically present more sickness-associated features in response to LPS (Cai et al., 2016; Kuo, 2016; Pitychoutis et al., 2009; Yee and Prendergast, 2010), potentially due to testosterone's role as an immunosuppressant and estrogen's role as an immune stimulator (Cai et al., 2016). Interestingly, opioids generally lead to suppression of gonadal hormones, including testosterone and estradiol (Seyfried and Hester, 2012). Thus, alteration of gonadal hormone levels may be one potential explanation for increased sickness scores in morphine-exposed female rats, such that lower estradiol levels in MOR female rats may induce male-typical sickness response. However, preliminary evidence from this model did not indicate any differences in anogenital distance (see Appendix B), timing of puberty, or estrous cycling (data not shown).

POE was also associated with altered cytokine levels post-LPS. Specifically, peripheral levels of IL-1α were significantly elevated in male and female MOR rats. IL-1α is a potent endogenous pyrogen (Netea et al., 2000), such that high circulating levels of IL-1α induce fever; in contrast, administration of IL-1 receptor antagonists block fever generation. IL-1α is generated in the periphery as a response to LPS stimulation, and is then transported via the

blood to the CNS, and in particular the median preoptic area (MnPO), to induce prostaglandin E2 (PGE2) synthesis (Netea et al., 2000). PGE2 then acts on EP3 receptors in the MnPO to induce downstream signaling to the raphe pallidus to promote heat generation through brown adipose tissue metabolism and vasoconstriction (Machado et al., 2020).

The increase in IL-1α observed in morphine-exposed male and female rats likely contributed to the increased febrile response in MOR rats, although the mechanism by which this occurs is currently unknown. IL-1α is primarily produced and released by peripheral macrophages in response to activation of the NF-κB transcription factor (Sharif et al., 2007). Previous studies have implicated opioids in modulation of NF-κB signaling (Wang et al., 2008; Welters et al., 2000); however, those studies report opioid-induced decreases in NF-κB and cytokine signaling rather than the increase noted here. It is possible that other cytokines and endogenous pyrogens (e.g., IL-1β, IL-10, TNFα) are altered during fever initiation and that the increase in IL-1α observed at 8 hours post-LPS is a response to other transient changes in cytokine levels. In the present studies, we only investigated peripheral cytokine levels, which are not always directly representative of the CNS. Future analysis of local cytokine levels in the MnPO may reveal subtle differences in other proinflammatory cytokines involved in fever generation.

4.5 The periaqueductal gray and immunity

In the present study, we also noted changes in microglial morphology, including decreased size and complexity, that are consistent with neuroinflammation in the periaqueductal gray (PAG). The PAG is an essential brain region for opioid-based analgesia due to its high μ-opioid receptor expression and downstream projections to the spinal cord (Doyle

et al., 2017; LaPrairie and Murphy, 2009). It is also an important brain region in opioid-induced immunosuppression (Gomez-Flores and Weber, 2000; Lysle et al., 1996; Weber and Pert, 1989). Our laboratory has previously reported that LPS administration to adult male and female rats results in a greater microglia response in females, as indicated by an increased proportion of non-ramified microglia; this increase in activation-associated morphology was accompanied by increased transcription of IL-1β (Doyle et al., 2017). This led us to hypothesize that markers of neuroinflammation would be increased in the PAG of rats exposed to morphine *in utero* in a sex-specific manner. We report here that microglia in the PAG of morphine-exposed male and female rats displayed increased activation-associated morphology. Deramification of microglial morphology is correlated with cytokine production (Althammer et al., 2020), which would also be predicted to increase the febrile response to LPS. Microglial phenotype was also analyzed in the entorhinal cortex, which has high μ-opioid receptor expression but no known connection to immunity. We observed results similar to the PAG, such that MOR rats had increased deramification and activation-associated morphology, suggesting that opioids act locally in the brain on μ-opioid receptors to increase microglial reactivity in response to LPS. Although the exact mechanism by which POE induces long-term increased microglial reactivity is unknown, one possible explanation is that morphine acts as a developmental stressor, which generates immune activation during microglia infiltration and maturation, leading to changes in reactivity and responsiveness (Carloni et al., 2021). Although opioids typically suppress peripheral immune cell activity, morphine is known to activate microglia in a TLR4-dependent manner (Doyle et al., 2017; Wang et al., 2012). Interestingly, LPS also binds to the TLR-4/MD-2 complex to promote neuroinflammation and cytokine production (Wang et al., 2012). This suggests that

increased microglial activity and cytokine production may provide an additional mechanism by which perinatal opioid exposure potentiates fever and sickness response, along with changes in peripheral immune function.

4.6 Perinatal opioid exposure and the gut

To determine the relationship between baseline peripheral immune function and the increased response observed in response to LPS, we also investigated gut permeability and antibody production. We first investigated gut permeability due to opioid's well-known role in generating "leaky gut" (Wang and Roy, 2017) due to damages in tight junction integrity (Meng et al., 2013). In the present study, morphine-exposed females had increased gut permeability, suggesting damage to the epithelial barrier that separates the gut lumen (where the microbiota is located) from the lamina propria (which contains the immune cells). A healthy and intact epithelial barrier serves to limit contact of bacteria and microbial products from the immune system in order to limit inflammation (Barbara et al., 2021). When the gut barrier is compromised, more bacteria can contact the immune cells in the lamina propria, leading to increased inflammatory signaling.

Increased gut permeability was limited to morphine-exposed females. Previous studies have implicated estrogen in increased gut epithelial cell turnover and proliferation (Sankaran-Walters et al., 2013), suggesting that female rats may be more sensitive to the negative impact of opioids on gut barrier function. Furthermore, opioids induce leaky gut in a TLR-dependent manner (Meng et al., 2013). Though it is unclear if there is a sex difference in the level of TLR expression on epithelial cells, differential levels of TLR expression between male and female rats would account for the sex-specific effect of POE on leaky gut. Importantly, there are sex-

and hormone-dependent differences in TLR expression on multiple immune system subtypes, although specific differences are often variable between cell types (Klein and Flanagan, 2016).

We anticipated that increased gut permeability would be associated with increased anti-LPS antibody levels. LPS, as a highly abundant molecule found on the surface of Gram-negative bacteria, is recognized by the immune system as a foreign antigen to induce antibody production. If the gut barrier is damaged, we hypothesized that more bacteria from the gut lumen would come into contact with immune cells in the lamina propria, leading to increased levels of anti-LPS antibodies. Surprisingly, we noted the opposite, such that anti-LPS antibody levels were significantly decreased in morphine-exposed rats. We theorized two potential explanations, the first being tolerance due to repeated exposure to bacterial antigens. Despite the presence of the gut epithelial barrier, immune cells in the lamina propria are constantly exposed to antigens from commensal bacteria. This can generate tolerance (Nutsch and Hsieh, 2012), which serves to minimize excessive inflammation. An additional hypothesis was an overall deficit in antibody production, which would lower anti-LPS antibody levels as well as immunoglobulins more generally. Therefore, in order to delineate the potential mechanism for decreased anti-LPS antibody levels, we measured levels of the three major antibody classes (IgG, IgA, and IgM). We found that morphine-exposed rats had lower levels of antibody, both IgG and IgM (females only), suggesting an overall decrease in antibody production. Deficits in antibody production can occur due to a variety of immunodeficiencies, including leukopenia, which is consistent with the increased fever response observed in response to LPS. Interestingly, we did note an increase in IgA antibody levels. IgA is important for coating commensal bacteria and limiting their translocation, suggesting that the immune system of

morphine-exposed rats attempted to counter the increased gut permeability by promoting agglutination, mucus production, and bacterial clearance (Bunker and Bendelac, 2018; Mantis et al., 2011). Furthermore, IgA serves as an immunological barrier, suggesting that MOR rats tried to ameliorate a damaged epithelial barrier by promoting IgA response (Tezuka and Ohteki, 2019).

Currently, the mechanism for decreased antibody production induced by perinatal opioid exposure is unknown. However, there are two potential, and not mutually exclusive options: 1) a decrease in the number of leukocytes, or 2) reduced performance of antigen-presenting cells (APCs). Opioid exposure in adults has been associated with both leukopenia (Flores et al., 1995; Hamra and Yaksh, 1996) and decreased function of APCs (Eisenstein et al., 1993); this suggests that perinatal opioid exposure may promote similar immune deficits that present as decreased antibody levels. Future studies should investigate the influence of POE on both the number and identity of white blood cells, as well as investigate antigen-induced antibody production in vitro to identify potential mechanisms by which this process is inhibited by morphine.

It is also important to note that gut permeability is associated with gut dysbiosis. This, along with morphine's known role in inducing gut dysbiosis, led us to investigate the gut microbiota composition of morphine-exposed male and female rats.

4.7 Gut microbiota dysbiosis alters immune system development and training

Opioids can act directly on white blood cells to decrease proliferation and induce leukopenia (Bayer et al., 1990; Flores et al., 1995). An additional mechanism whereby opioids can influence the immune system is the induction of gut microbiota dysbiosis, or an altered

composition of operational taxonomic units (OTUs) that make up the microbiota. The microbiota serves as an essential immune stimulator during critical periods of early development, and dysbiosis during this period can have a long-term impact on the immune system's maturation (Stiemsma and Michels, 2018). Composition of the gut microbiota regulates the development of both the innate and adaptive immune system, including hematopoiesis (Wu and Wu, 2012; Yan et al., 2018). In the current study, we investigated alpha diversity (the richness and evenness of the gut microbiota within a sample), beta diversity (the differences in composition between samples), and changes in individual operational taxonomic units (OTUs) that make up the microbiota in both dams and offspring. Perinatal exposure to morphine produced robust gut microbiota dysbiosis by altering beta diversity and OTU makeup. Increased gut permeability was also observed in females. Exposure of the immune system to dysbiotic microbiota, along with increased bacterial contact due to leaky gut, could alter the development of the immune system by promoting tolerance and/or sensitization to bacterial antigens, including LPS. Presently, we report decreased (rather than increased) anti-LPS antibody levels; thus, it is possible that repeated exposure to LPS and other bacterial antigens induced tolerance in innate and adaptive immune responses. However, as we also identified decreased total IgG and IgM levels, along with elevated fever response to LPS treatment, it is unlikely that immunotolerance is the primary mechanism responsible for these effects in morphine-exposed rats. Instead, it is more plausible that overall immune system function was dampened as a result of morphine exposure (both directly and indirectly via the microbiota).

Maternal microbiota composition is the primary determinant of offspring microbiota (Dominguez-Bello et al., 2010). Thus, it is surprising that only minor differences in beta diversity

were observed between morphine-treated and control dams, despite the large differences observed in their offspring. The limited effects of morphine treatment on dam bacterial composition may be due to the more dominant effects of pregnancy. In support of this, the only significant differences in OTU composition for VEH and MOR dams occurred before the initiation of breeding. Pregnancy leads to a dramatic restructuring of gut microbiota composition, which is theorized to be associated with changes in maternal body weight and metabolism, as well as acting to promote fetal health and development (Edwards et al., 2017). Pregnancy also creates a generalized proinflammatory state, which in a non-pregnant person would be a marker of disease, but is necessary for metabolic changes in the mother, as well as fetal health (Edwards et al., 2017).

4.8 Altered gut microbiota diversity in morphine-exposed offspring

Despite limited changes in OTU abundance observed in morphine-treated dams, male and female rats exposed to morphine *in utero* had long-term and robust alterations in gut microbiota composition. Only two OTUs were significantly altered in dams; however, 10-19 OTUs were altered in offspring from P21-P70. The majority of these OTUs were members of the families Lachnospiraceae, Ruminococcaceae, and S24-7. These families are highly prevalent members of the rat gut microbiota, and are involved in bile acid and short-chain fatty acid (SCFA) production, major microbial metabolites involved in immune function and gut microbiota composition. Healthy microbiota should produce bile acids and SCFAs to help maintain intestinal barrier function and promote intestinal immunity. However, in previous work, the opioid-exposed microbiota decreased production of both bile acids and SCFAs due to loss of producers of these metabolites (Banerjee et al., 2016; Cruz-Lebrón et al., 2021; Wang et

al., 2018). Alteration of these families, particularly during early life, likely contributed to the altered immune system development observed in morphine-exposed rats, as bile acids and SCFAs are essential in the maturation of the intestinal immune system (Wang et al., 2018).

We also investigated the maturation of the gut microbiota in vehicle vs. morphine-exposed rats. In adulthood, the maturity and predicted age of the morphine-exposed microbiota were significantly lower than vehicle rats. Decreased microbial maturity has been reported in malnourished children and premature children in the NICU (Subramanian et al., 2014; Yee et al., 2019), suggesting that perturbations in early life lead to long-term alterations in microbial composition. Importantly, nutritious food intervention did not improve microbial maturity in malnourished children, suggesting that microbial immaturity is maintained long after the cessation of early life perturbations (Subramanian et al., 2014). This highlights the importance of early life interventions to potentially ameliorate gut microbiota dysbiosis in children exposed to opioids *in utero*.

When comparing beta diversity, sex differences were generally overshadowed by the effects of morphine. Previous studies have reported mixed effects of biological sex and sex hormones on gut microbiota composition, which are complicated by other confounding factors such as diet, body mass, and medication (Kim et al., 2020). In the present study, the effect of morphine on individual OTUs was sex-specific, but were generally members of the same families (mainly Lachnospiraceae, Ruminococcaceae, and S24-7).

The microbiota has a critical role in immune system maturation during early life, when the microbiota is most vulnerable to environmental insults (Zheng et al., 2020). Dysbiosis of commensal bacteria can lead to long-term consequences in immune system development,

including excessive inflammatory response to TLR stimulation in the gut, autoinflammatory disease, increased susceptibility to gastrointestinal pathogens, and altered cell-based innate and adaptive immune responses (Belkaid and Hand, 2014; Zheng et al., 2020). This has led us to form a theoretical model of the effects of perinatal opioid exposure on gut microbiota dysbiosis and immunity (see Figure 4-2). Perinatal exposure to opioids, along with inheritance of a dysbiotic microbiota from opioid-exposed mothers, induces gut dysbiosis and increased gut permeability. This allows for differential immune system development as a result of exposure to proinflammatory and pathogenic microbes, ending in increased susceptibility, morbidity, and mortality to infection.

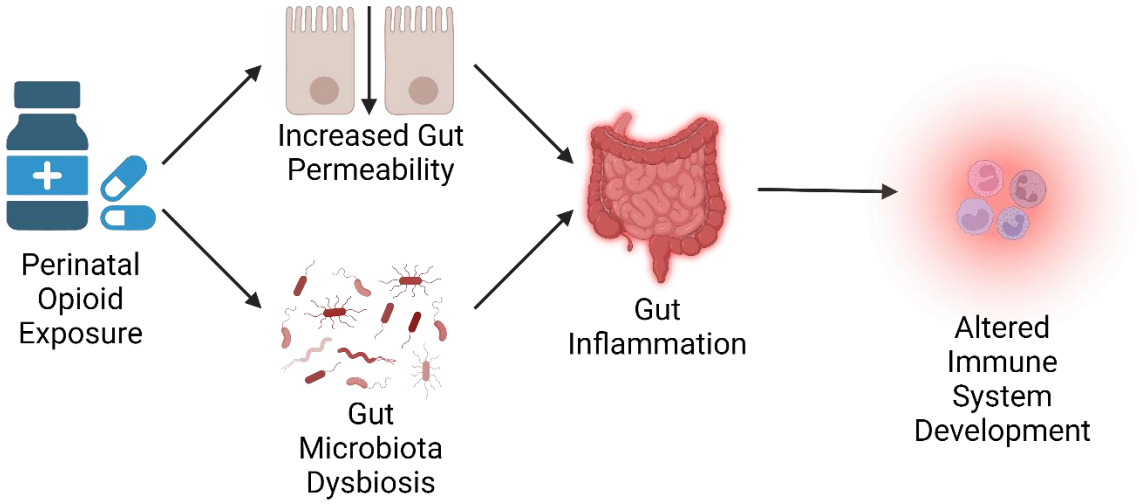


Figure 4-2. Proposed model of the effect of perinatal opioid exposure on the microbiota and subsequent immune function. Exposure to opioids induces leaky gut and gut dysbiosis, which promotes gut inflammation. Exposure of the immune system to the dysbiotic gut microbiota during critical windows of immune system maturation leads to long-term alterations in immune function, promoting susceptibility to pathogens as a result of altered development.

4.9 Future directions to improve the quality of life of children exposed to opioids *in utero*: Focus on the immune system

Opioids are undeniably an essential pharmacological tool for the treatment of pain; however, overuse and abuse by women of reproductive age has the potential to not only negatively affect their quality of life, but that of the next generation as well. The long-term health impacts of children exposed to opioids *in utero* are largely unknown, despite the fact that approximately 22,000 infants are born with neonatal opioid withdrawal syndrome every year. No treatments or interventions exist for these infants, apart from initial supplemental opioid therapy to minimize withdrawal. The potential additional health burden of immunodeficiencies and gut microbiota dysbiosis adds to the already heightened healthcare expenditures in this population (Malthaner et al., 2022; Uebel et al., 2015; Witt et al., 2017).

Moving forward, one thing is clear: there must be more clinical studies on the long-term effects of neonatal opioid withdrawal syndrome and *in utero* opioid exposure, particularly regarding immunity. The limited evidence available suggests that *in utero* opioid exposure promotes immunosuppression and gut microbiota dysbiosis, but the clinical implications and significance of those findings is largely unknown. By characterizing the immune deficits associated with perinatal opioid exposure, those infants can be targeted for early screening and intervention. Although treatment for immunodeficiency is limited, if children exposed to opioids *in utero* are leukopenic or have antibody deficiencies, they may be eligible for immunoglobulin or growth factor treatments to improve health outcomes. Furthermore, gut dysbiosis could potentially be corrected or ameliorated by probiotic treatment or fecal

transplant, although evidence is currently mixed on the efficacy and safety of those interventions, particularly in children.

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5 APPENDIX A - EARLY LIFE OPIOID EXPOSURE AND POTENTIAL LONG-TERM EFFECTS

5.1 Introduction

In 2016, physicians wrote 66.5 prescriptions for opioid-based medications per 100 people in the United States (Centers for Disease Control, 2017), of which an estimated 4.7 persons misused their prescription. Although opioid prescribing rates have leveled off since 2012, due in part to increased caution on the part of healthcare providers, opioids are still prevalent drugs of abuse, and both prescription pain relievers and commonly abused drugs like heroin and fentanyl are a significant source of drug abuse and mortality. Indeed, in 2015, opioids accounted for 63.1% of all overdose deaths in the U.S. (Centers for Disease Control, 2017).

Women are prescribed opioids at a higher rate than men, with an average of 21.8 patients per 100 women in comparison to 16.4 per 100 men (Centers for Disease Control, 2017). Between 2008 and 2012, approximately 28% of privately-insured and 39% of Medicaid-insured women of childbearing age (15–44 years) filled a prescription for an opioid (Ailes et al., 2015). As half of all U.S. pregnancies are unplanned, and pregnancy is often unrecognized until the sixth gestational week (American Pregnancy Association), women of childbearing age who use opioids, either prescribed or illicit, are at risk of exposing their fetus during a critical period of development (Ailes et al., 2015).

The rate of opioid prescriptions is remarkably high for pregnant women, with an estimated 14–22% of pregnant women filling a prescription for an opioid during their pregnancy (Ailes et al., 2015). Over the past 10 years, the rate of pregnant women who are dependent on opioids has steadily increased in the US, with an average of 0.9% of pregnant

women aged 15–44 having misused opioids in the last month (Smith and Lipari, 2017). Opioid misuse during pregnancy significantly jeopardizes the health and well-being of the developing infant. Indeed, approximately 60–80% of infants exposed to opioids *in utero* will experience neonatal opioid withdrawal syndrome (NOWS) following birth (Patrick et al., 2012). In the US, the incidence rate of NOWS has increased over 400%, from 1.2 per 1000 hospital births in 2000 to 5.8 in 2012; that statistic translates to approximately one NOWS infant born every 25 min (Patrick et al., 2015).

These statistics highlight the impact of the opioid epidemic on women of child bearing years and their offspring. A 400% increase in the incidence rate of NOWS is frightening, particularly given the dearth of clinical information regarding the long-term consequences. This review will provide an overview of the recent clinical and preclinical findings on perinatal opioid exposure, and when possible, its impact on stress-responsive circuits.

5.2 Clinical Studies on Perinatal Opioid Exposure

Infants exposed to opioids during gestation are more likely to be born prematurely (<37 weeks gestation) and at a lower birth weight (<2500 g) (Fill et al., 2018; Hunt et al., 2008). As stated above, approximately 60–80% of these infants will experience NOWS following birth (Patrick et al., 2012). Infants undergoing NOWS will experience many, if not all, of the same symptoms experienced by adults undergoing opioid withdrawal. These symptoms include decreased sleep, tremors, seizures, increased muscle tone, sweating and fever, gastrointestinal dysfunction including loose/watery stools and vomiting (Ainsworth, 2014). NOWS infants are also known for their continuous high-pitched crying and inability to be consoled (Ko et al., 2016). Reduced brain volume and increased risk of sudden unexpected death have also been

reported (Ko et al., 2016; Patrick et al., 2012). Secondary complications associated with NOWS include tachypnea, meconium aspiration, respiratory distress, jaundice, and sepsis (Patrick et al., 2015). Infants undergoing NOWS are more likely to be admitted into the neonatal intensive care unit (NICU) where they will spend an average of 17–23 days with an associated cost of $66,700-$93,000, depending on the need for pharmacological treatment (Ko et al., 2016; Patrick et al., 2012, 2015). In contrast, the average hospital stay for a full term infant is 2 days with an associated cost of $3500 (Patrick et al., 2015).

5.2.1 Clinical Outcomes

Although limited data are available regarding the impact of perinatal opioid exposure on behavioral outcomes, recent clinical studies have identified a number of cognitive, motor and sensory deficits. Children with a history of NOWS have significantly lower cognitive and motor performance scores in early childhood (Hunt et al., 2008; Baldacchino et al., 2014), and are more likely to be diagnosed with learning disabilities including developmental delays and/or speech and language disorders than age and demographic matched controls (Maguire et al., 2016; Fill et al., 2018). Although the underlying mechanisms regarding the cognitive deficits are unknown, studies in rodents have reported that *in utero* opioid exposure significantly attenuates both neural- and glial-genesis (Sanchez et al., 2008; Robinson, 2002), and decreases dendritic length and branch number in somatosensory cortical neurons (Lu et al., 2012). These preclinical findings are consistent with reports of reduced brain volumes in the basal ganglia, thalamus and cerebellar white matter in school-aged children exposed to opioids perinatally (Sirnes et al., 2017). Prenatal exposure to methadone is also associated with decreased

microstructure in white matter tracts in neonates (38–39 weeks of age), indicative of less organized and more immature fiber tracts (Monnelly et al., 2018).

Very few studies have assessed for behavioral outcomes in NOWS infants. In a cohort of 5–8 month olds, Bakhireva et al. (2019) reported higher negative affect and lower self-regulation in NOWS infants versus healthy controls. NOWS infants were also more likely to be rated as 'sensation seeking', i.e., are more likely to search for additional sensory stimulation via oral (biting/mouthing) or physical (touching) means. Mother-child interactions are also more likely to be rated as negative for NOWS infants (Konijnenberg et al., 2016). Specifically, mothers with opioid use disorder (OUD) during pregnancy displayed lower levels of sensitivity, expressed less positive affect, and engaged in few activities with their offspring at both 12 months and 4 years of age. In parallel, children with a history of NOWS demonstrated lower positive affect, less interest in activities, and less involvement with those around them in comparison to age-matched controls (Konijnenberg et al., 2016). In addition to the reduced maternal-infant bond observed in NOWS infants, women with OUD during pregnancy are more likely to be single, have less high school education, lower family socioeconomic status, and have additional children (Bakhireva et al., 2019); these factors have been shown to contribute to the increased levels of maternal stress, which is associated with reduced maternal care.

5.2.2 Causal Factors

The studies reviewed above indicate that infants born with NOWS are at an increased risk for neurodevelopmental impairments and behavioral difficulties. The impact of perinatal opioid exposure in these infants is further compounded by the lack of pre- and postnatal healthcare, poor maternal diet, and increased incidence of premature birth (Fill et al., 2018).

Polydrug use is also common in pregnant women with an OUD, who consume, on average, 3.3 different drugs of abuse, including tobacco, alcohol, cocaine and benzodiazepines (Nygaard et al., 2016; Sirnes et al., 2017; Monnelly et al., 2018).

5.2.3 Opioid Pharmacokinetics

The specific mechanisms whereby perinatal opioid exposure results in changes in brain structure and/or function are not known; indeed, it is likely a combination of factors (see above) in addition to the opioids that contributes to the overall phenotype. Importantly, there are significant differences in the pharmacokinetic profile of morphine for infants in comparison to later developmental stages that may amplify the negative effects of opioid exposure. For example, in infants, the half-life of morphine is 6–12 h, compared to approximately 1 h in 1- to 6-year olds (Ainsworth, 2014). This prolonged duration of action is due to a slower elimination rate, as morphine clearance does not reach adult levels until two weeks to six months of age (Ainsworth, 2014; McRorie et al., 1992; Lynn and Slattery, 1987). Morphine also remains largely unbound during the first few weeks of life, leading to high sensitivity and increased accumulation in the brain (Bhat et al., 1990), an effect accentuated by the underdeveloped blood-brain barrier (Vathy, 2002). Morphine is metabolized via glucuronidation, which is functional at both the 3 and 6 positions in infants to produce morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), respectively (Choonara et al., 1992). However, due to an underdeveloped hepatic glucuronidation system, infants are less able to metabolize morphine to M6G, the functional metabolite of morphine, and therefore have higher levels of the M3G metabolite (Hartley et al., 1994; Bouwmeester et al., 2004). M3G has little to no affinity for the μ opioid receptor (MOR), but rather has a high affinity for the innate pattern receptor toll-like

receptor 4 (TLR4). Results from preclinical studies suggest that morphine signaling via TLR4 contributes to many of the negative side effects associated with opioid consumption, including opioid-induced hyperalgesia and tolerance (Due et al., 2012; Eidson and Murphy, 2013; Eidson et al., 2017), although this relationship remains controversial (Mattioli et al., 2014; Fukagawa et al., 2013). Regardless, the combination of long half-life, accumulation in the brain, and altered metabolism patterns suggests that infants are especially at risk for long-term developmental consequences of opioid exposure.

5.2.4 Endogenous Opioid Levels

During pregnancy, plasma levels of β-endorphin increase from approximately 20 fmol/mL to >120 fmol/mL by week 6, where they remain until parturition (Panerai et al., 1983). By postnatal day 5, endogenous opioid levels in both the infant and mother decrease to normal adult levels (Manfredi et al., 1983; Panerai et al., 1983). Infants exposed to opioids *in utero* also show elevated levels of β-endorphin, however, rather than decreasing to normal levels following birth, β-endorphin levels continue to increase to approximately 100x higher than age-matched controls (Manfredi et al., 1983; Panerai et al., 1983). Both β-endorphin and met-enkephalin levels remain elevated in opioid-exposed infants at postnatal day 40 (Manfredi et al., 1983; Panerai et al., 1983). Interesting, although all of the infants in the Manfredi study developed NOWS and required medication-assisted therapy, there was no effect of treatment (paregoric or phenobarbital) on endogenous opioid levels (Manfredi et al., 1983). Similarly, there was no relationship between NOWS symptom severity and endogenous opioid plasma levels.

Mu opioid receptor (MOR), the primary receptor for the exogenous opioids morphine, oxycodone, and heroin, and the endogenous opioid B-endorphin, is detectable in the CNS as early as 12–13 weeks' gestation (Ray and Wadhwa, 1999). This indicates that there is functional MOR present in the CNS while the infant is exposed *in utero* to high levels of not just illicit opioids, but also B-endorphin. Endogenous opioids modulate CNS development by primarily inhibiting growth (Hauser et al., 1989; Zagon et al., 1982; Zagon and MacLaughlin, 1987), providing an additional mechanism underlying the decrease in brain volume observed in NOW infants.

Previous studies in rodents have implicated endogenous opioids and their receptors in long-term cognitive deficits. In rats, early life pain (postnatal day 0), which significantly increases brain B-endorphin levels (Victoria et al., 2015) significantly impedes memory recall in rats tested as adults in the Morris Water Maze (Henderson et al., 2015). Administration of naltrexone, a non-selective opiate receptor antagonist, at the time of injury improved long-term memory on the radial arm water maze in adulthood (Nuseir et al., 2017). Together, this data suggests that elevated levels endogenous opioids in the brain may further contribute to the long-term deficits in cognition observed in NOWS infants.

5.3 Opioids and Stress

As discussed above, remarkably little is known regarding the negative behavioral outcomes associated with perinatal opioid exposure in humans. In adult rodents, acute or chronic opioid exposure activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of corticotropin-releasing hormone (CRH), which then acts on the pituitary to stimulate adrenocorticotropic hormone (ACTH) release. ACTH stimulates the release of

corticosterone in rodents or cortisol in humans (CORT) from the adrenal gland (Ignar and Kuhn, 1990), which then feeds back to the hypothalamus to turn off the further release of CRH in a negative feedback manner. Although the impact of perinatal opioid exposure on CORT release is not known, chronic morphine exposure in adults leads to tolerance of the CORT-releasing effects of morphine (Houshyar et al., 2001).

Studies in rodents have reported long-term changes in the hormonal and behavioral responses to acute and chronic stress following perinatal opioid exposure. For example, administration of morphine to rats on the day of birth results in significant anxiolytic behavior in the open field apparatus and forced swim test in adulthood (Victoria et al., 2015). Reduced basal CORT levels and blunted CORT response following 10 min of forced swim have also been reported (McPherson et al., 2007; Victoria et al., 2013, 2014). In contrast to the hypo-responsive profile observed following exposure to acute anxiety- and/or stress-provoking stimuli, the exact opposite behavioral response (hyper-responsive phenotype) was observed in P0 morphine treated rats following prolonged exposure (7 days) to mild chronic variable stress in adulthood. Similar changes in the behavioral response to stress have also been reported in adult rats born to dams who received morphine during gestation (Ahmadalipour and Rashidy-Pour, 2015; Ahmadalipour et al., 2015; Šlamberová et al., 2002; Haydari et al., 2014; Fodor et al., 2014). Together, these studies suggest that early activation of the opioid system produces long-term changes in the neurocircuitry underlying stress (LaPrairie and Murphy, 2009; Victoria et al., 2015). Interesting, enhanced morphine reward, as indicated by increased morphine preference during the two-bottle choice paradigm, was reported in adult male rats exposed to

opioids *in utero* (Haydari et al., 2014), suggesting an increased propensity for drug abuse in adulthood.

5.4 Potential Interventions

Researchers have now begun to identify potential, non-pharmacological interventions to mitigate the effects of prenatal opioid exposure. One promising intervention is exercise during pregnancy. Previous studies have reported that exercising dams improves outcome measures following naloxone precipitated withdrawal, and in the offspring, decreases anxiolytic behavior and morphine preference in a two-bottle choice paradigm (Haydari et al., 2014). Exercising pups from P21-P40 also attenuated responses to stress as indicated by improved time in the light compartment and open arm of the elevated plus maze (Ahmadalipour and Rashidy-Pour, 2015). Providing environmental enrichment to pups resulted in similar improvements, and also decreased hippocampal BDNF levels (Ahmadalipour et al., 2015). Co-administration of naloxone with opioids during pregnancy attenuated most negative effects, suggesting that the impact of perinatal opioid exposure is largely mediated via MOR signaling (Vathy, 2002).

Clinically, infants successfully treated for NOWS may show better outcomes than those who experienced uncontrolled withdrawal in early life (Vathy, 2002). Maternal factors and genetic background are likely contributing factors, as opioid use disorder is highly heritable and polymorphisms in OPRM1 and COMTare associated with shorter length of hospital stay and decreased the likelihood of pharmacological treatment after prenatal opioid exposure (Wachman et al., 2013; Lesage et al., 1998). Together, this suggests that early life interventions targeted to the mother or infant, along with traditional pharmacological treatment, have the potential to mitigate long-term complications due to NOWS, and that individual factors of drug

use and genetic profiles should be factored in to create individualized treatment plans in the NICU.

5.5 Future Concerns

Although a growing body of evidence suggests that early life opioid exposure can lead to long-term alterations in behavior and neurochemistry, the wide range of covariates in clinical data makes interpretation difficult. Particularly important for clinical translation is the observation that most preclinical models administer opioids to pregnant dams from E11-18; this development time point coincides with the emergence of androgen, estrogen, and endogenous opioid systems (Vathy et al., 1985), and is a critical period of CNS sexual differentiation (Vathy and Katay, 1992) (see Table 1). Other studies administer morphine from E5-12, which coincides with organogenesis and approximates the first trimester of human development, when infants are most sensitive to teratogens (Vathy et al., 1983, 1985; Vathy and Katay, 1992). Several studies also employ an increasing dose paradigm, where the first three doses (in these cases, on E5 and E6) were 5 mg/kg, and the remaining doses (from E6-12) were 10 mg/kg (Litto et al., 1983; Vathy et al., 1983). Importantly, these dosing paradigms do not accurately model the clinical profile of the prototypical female with OUD who typically initiates opioid consumption prior to pregnancy. As a newborn rat pup is considered neurodevelopmentally comparable to a third trimester infant, the ability to translate the results of E11-18 dosing schedule to clinical outcomes in infants born with substance use disorder is in doubt. Drug use is rarely initiated in pregnancy; rather, a pregnant woman is more likely to have initiated illicit drug use in adolescence and to actually decrease drug use over pregnancy. Indeed, only 2.4% of pregnant women report misusing drugs in the third trimester versus 9.0%

in the first trimester (Creasy et al., 2014; Substance Abuse and Mental Health Services Administration, 2014). Future preclinical studies should attempt to utilize a dosing schedule that more accurately reflects actual use patterns of pregnant women, thereby improving translatability of the results.

A recent study by Byrnes and Vassoler (2018) addresses many of the concerns identified above. In this study, adult female rats were trained to self-administer oxycodone prior to and during gestation. Similar to what is observed clinically, dams who self-administered oxycodone during gestation showed reduced maternal responding (as indicated by longer latencies in the pup retrieval test), and their pups weighed significantly less at birth. In contrast to NOWS infants, pups born to oxycodone dams did not show an increase in ultrasonic vocalizations (indicative of distress), although it is not clear whether these pups displayed symptoms consistent with opioid withdrawal. Clearly, additional preclinical research needs to be conducted to delineate the long-term consequences of perinatal opioid exposure, and the study by Byrnes and Vassoler epitomizes the type of model design that should be employed in future pre-clinical studies; however, dosing should be refined such that clinically relevant withdrawal symptoms appear in the offspring of opioid-exposed dams.

5.5.1 Clinical Relevance

The confounding factors of poor prenatal care, poor nutrition, concomitant drug use, and low maternal care make interpretation of clinical data challenging. Preclinical data show that morphine-injected dams eat approximately 25 percent less food than saline-injected controls (Kirby, 1983). When non-injected controls were pair-fed to morphine-injected dams, causing them to be food restricted, pups born to pair-fed dams were born at lower birth

weights, similar to morphine-exposed pups; both cohorts reached normal weight by P6 (Kirby, 1983). Pair-fed and morphine-exposed pups also showed a reduction in spinal volume, in particular gray matter volume, suggesting that morphine's growth-retardant effects on CNS development are due, in part, to reduced maternal food consumption (Kirby, 1983). The fact that spinal volume was still decreased at P6 when body weight had returned to normal levels suggests that the spinal cord (and likely the entire CNS) is permanently impacted (Kirby, 1983). Future clinical studies may wish to include metabolic and nutritional factors in their analysis, as well as considering dietary interventions during pregnancy to improve mother and infant's quality of life.

5.5.2 Medication-Assisted Therapies

Although this review has thus far focused on morphine as the prototypical opioid, it is important to note that opioid-dependent mothers have often transitioned onto maintenance doses of methadone or buprenorphine when their pregnancy is discovered. Methadone has been the gold standard of treatment for pregnant women, but recent data suggests that buprenorphine may be a better choice to manage opioid dependency during pregnancy. For example, some studies report that methadone-exposed infants have an increased risk of developing NOWS as compared to buprenorphine-exposed (Lemon et al., 2018), and buprenorphine-exposed infants may require a lower dose and shorter duration of pharmacological treatment (Jones et al., 2005, 2010; Tolia et al., 2018; Hall et al., 2016), as well as a shorter length of hospital stay (Kraft et al., 2017). However, one study found more women discontinued the use of buprenorphine during their pregnancy, citing ‘dissatisfaction’ with the medication (Jones et al., 2010). The effect on NOWS severity is unclear, as some studies report

no difference in peak NOWS scores (Jones et al., 2005) and others report higher mean severity for methadone-exposed infants versus buprenorphine (Gaalema et al., 2012). Methadone-exposed infants may also require pharmacological treatment earlier in life, with NOWS symptoms of undisturbed tremors and hyperactive Moro reflex increased in prevalence and severity (Gaalema et al., 2012). A longitudinal study on methadone- and buprenorphine-exposed infants found very little differences in developmental and behavioral outcomes at 3 years, suggesting that both drugs may serve as appropriate treatments during pregnancy (Kaltenbach et al., 2018).

5.6 Conclusion

Improving our animal models of NOWS will allow for a more clear delineation of the long-term deleterious consequences of perinatal opioid exposure, and develop new interventions to improve the quality of life of infants born with NOWS. This research can also be translated to the bedside, by informing clinical prescribing patterns and maternal interventions to prevent future deleterious consequences to their offspring.

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Study Name	Morphine Dose/Exposure Time	Pup treatment?	Age at testing?	Main effects?	Interventions?	Citation for E11-18?
Ahmadalipour and Rashidy-Pour (2015)	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half-saline and half morphine-exposed pups. Litters reduced to 10 maximum. Sexes of pups not tested; sex not considered as a biological variable.	Tested between P41-47.	Less time in light compartment of light/dark box. Less OAT% for EPM.	Pups were exercised 30 min per day from P21-40. Improved TLC and OAT%.	Vathy et al. (1985)
Ahmadalipour et al. (2015)	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half-saline and half morphine-exposed pups. Litters reduced to 10 maximum, with an equal sex balance. One saline-exposed and one morphine-exposed pup per sex per dam was selected for behavioral testing. Data collapsed across sexes due to high Pearson's correlation.	Tested between P51-57.	Smaller time in light compartment/entries into light compartment on L/D box. Lower OAT on EPM. Lower STL. Higher TDC. Lower BDNF levels.	Enrichment from P21-50. Improved OAT, STL, TDC, and BDNF.	Vathy et al. (1985)
Laborie et al., 2005	E11-18 2x daily 10 mg/kg. SubQ.	Litters reduced to 10 maximum. Only male rat pups tested.	Tested at 3 months age.	Decreased basal adrenal NE content. Lower basal adrenal PNMT, which did not respond to ether stress. Increased hippocampal 5HT/5H1AA after ether stress. Increased hypothalamic basal 5HT/5H1AA. Increased hypothalamic 5H1AA after stress.	Maternal adrenalectomy improves HPA axis function.	Vathy et al. (1985) and Lesage et al. (1998)

Lesage et al. (1998)	E11-18 2x daily 6 mg/ml (20 mg/kg/day). SubQ.	No differences seen between male and female pups; pups pooled. Used 2–4 pups from some of the mothers in each group for experiments.	Tested at P0.	Adrenal atrophy/hypoactivity. Reduced CRF in the hypothalamus.	Maternal adrenalectomy at E10 minimizes effects of maternal opiate exposure on the HPA axis.	N/A
Rimanóczy and Vathy, 1995	E11-18 2x daily. First 2 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half saline and half morphine-exposed pups. Litters adjusted to 8–10 pups. One male and one female from each litter used. All females OVX.	OVX ~ 7–10 days before sacrifice. Tested at P75-85.	Estradiol only affects hypothalamic Bmax of mu opioid receptors in morphine exposed OVX females.	N/A	Vathy and Katay (1992) (cites Vathy et al., 1985) and Vathy et al., 1994 (cites Vathy et al., 1983; Vathy et al., 1985; Vathy and Katay, 1992)
Rimanóczy et al., 2003	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half-saline and half morphine-exposed pups. Litters reduced to 10 maximum, with an equal sex balance. One saline-exposed and one morphine exposed male pup per litter was selected for analysis.	Tested between P60-90.	Smaller stress-induced increased in ACTH for males.	N/A	Vathy et al. (1983) and Vathy et al. (1985)

Šlamberová et al., 2004	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half-saline and half morphine-exposed pups. Litters reduced to 10 maximum. One saline-exposed and one morphine exposed female pup per litter was selected for analysis.	Tested between P60-90.	Smaller stress-induced increase in ACTH.	N/A	Vathy et al. (1985)
Šlamberová et al. (2002)	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half-saline and half morphine-exposed pups. Litters reduced to 10 maximum, with an equal sex balance. One saline and one morphine-exposed male or female used from each litter.	OVX at P60. Tested between P70-90.	Males showed more struggling and less swimming on the forced swim, as well as an increased number of squares visited along walls after cold water stress. OVX females showed less grooming on the open field.	N/A	Vathy, 1995; Vathy et al. (1985)
Vathy et al., 2000	E11-18 2x daily. First 3 injections 5 mg/kg, remaining injections 10 mg/kg. SubQ.	Cross-fostered so each mother raised half saline and half morphine-exposed pups. Litters adjusted to 10 pups. Both males and females used. All females OVX.	OVX at P60. Tested at P65-75.	Increased TH-IR in male caudal PVN/LC. Decreased TH/IR in female LC. Estradiol does not affect LC TH-IR in morphine-exposed females.	N/A	Vathy and Katay (1992) (cites Vathy et al., 1985)

Table A. 1. Summary of studies utilizing E11-18 dosing schedule.

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6 APPENDIX B – PERINATAL OPIOID EXPOSURE LEADS TO DECREASED SOCIAL PLAY IN ADOLESCENT MALE AND FEMALE RATS: POTENTIAL ROLE OF OXYTOCIN SIGNALING IN BRAIN REGIONS ASSOCIATED WITH SOCIAL REWARD

6.1 Introduction

The dramatic rise in maternal opioid use disorder in the United States over the last two decades has led to significant increases in the number of infants born with neonatal opioid withdrawal syndrome (NOWS) (Hirai et al., 2021). NOWS infants experience more complicated births and extended stays in the hospital, typically 9 days longer than non-exposed infants (Hirai et al., 2021). Although limited data are available regarding the impact of perinatal opioid exposure on behavioral outcomes, clinical studies have identified several cognitive, motor, and sensory deficits. Children with a history of NOWS have significantly lower cognitive and motor performance scores in early childhood (Baldacchino et al., 2014; Hunt et al., 2008) and are more likely to be diagnosed with learning disabilities than age- and demographically-matched controls (Fill et al., 2018; Maguire et al., 2016). Clinical literature further suggests *in utero* opioid exposure leads to long-lasting deficits in sociability. More specifically, children who were exposed to opioids prenatally score higher on assessments for autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (Sandtorv et al., 2018). These observed deficits are especially prevalent in the “social difficulties” subscale, which assesses prosocial behavior, including the ability to form friendships, and social communication (Sandtorv et al., 2018). Similar studies investigating the impact of *in utero* opioid exposure also identified lower levels of social maturity during early childhood (Hunt et al., 2008).

Juvenile play is observed in most mammalian species and is essential for normal social development (see Bredewold and Veenema, 2018 or Veenema, 2012 for review). Participating in social play during adolescence facilitates the development of behavioral patterns that provide the framework for response to future challenges (Achterberg et al., 2019; Hol et al., 1996; Manduca et al., 2014). Indeed, isolation of rats during the juvenile period leads to deficits in adult social behavior, including decreased social motivation (Van Den Berg et al., 1999a), anogenital sniffing (Van Den Berg et al., 1999b), and social approach (Van Den Berg et al., 1999b; Van Den Berg et al., 2000). Therefore, social play is considered an indicator of general sociability, with adolescence being a critical period for its development.

Neuropharmacological studies utilizing acute opioid exposure in adulthood support a clear role for opioids in social attachment: systemic administration of μ-opioid receptor agonists enhances social play in male and female rats (Manduca et al., 2014; Vanderschuren et al., 1995a; Vanderschuren et al., 1995b; Vanderschuren et al., 1995c), and this effect is blocked by co-administration of μ-opioid receptor antagonists (Beatty and Costello, 1982; Jalowiec et al., 1989; Normansell and Panksepp, 1990; Siegel and Jensen, 1986; Siegel et al., 1985; Trezza et al., 2011). Similarly, administration of the μ-opioid receptor-selective antagonist CTAP into the caudate putamen of female prairie voles completely inhibits the formation of a partner preference (Burkett et al., 2011). In contrast, preclinical studies investigating the impact of perinatal opioid treatment on social play are limited and often contradictory, with reports of increased (Buisman-Pijlman et al., 2009; Hol et al., 1996; Niesink et al., 1999), decreased (Chen et al., 2015), and no effect (Minakova et al., 2022) on social behavior. These contradictions are likely due to differences in both the stage of gestation in which opioid administration is initiated

and the duration of exposure. For example, in the above-mentioned studies, administration paradigms range from daily injections beginning on day 8 of pregnancy [E8] and continuing to parturition (Buisman-Pijlman et al., 2009) to exposure from E3-E20 (Chen et al., 2015); still others administer opioids from E0 until weaning at postnatal day 21 [P21] (Minakova et al, 2022). Importantly, these opioid administration paradigms fail to recapitulate the clinical profile of maternal opioid use, which typically begins in late adolescence, *before* pregnancy, and decreases in the final weeks before birth (Smith and Lipari, 2017). Thus, although clinical studies have linked gestational opioid exposure to deficits in sociability, there is a clear need for the development of more translational preclinical models to begin to address the underlying neural mechanisms.

The neuropeptide oxytocin (OT) has been implicated as a critical neuromodulator of social behavior (Bredewold et al., 2014; Rigney et al., 2022). For example, oxytocin signaling within the nucleus accumbens facilitates social reward in mice (Choe et al., 2022; Dölen et al., 2013), rats (Smith et al., 2017), and prairie voles (Johnson et al., 2017; Keebaugh et al., 2015; Keebaugh and Young, 2011), and OT+ Fos expression in the supraoptic nucleus (SON) is positively correlated with the percent of time juvenile male and female rats engaged in play (Reppucci et al., 2018). Critically, chronic opioid exposure in male rats leads to decreased oxytocin levels in the SON (Laorden et al., 1997) and blood (Van de Heijning et al., 1991) due to direct inhibition of OT synthesis by μ-opioid receptor agonists, including morphine (Li et al., 2001). Together, these studies suggest that developmental exposure to opioids may attenuate sociability by suppressing OT expression and/or signaling, however, to date, this has not been examined.

The present studies tested the hypothesis that perinatal morphine exposure leads to alterations in juvenile play via changes in OT peptide expression. Importantly, these studies are the first to use a clinically relevant rodent model in which morphine is administered prior to, during, and immediately following parturition to investigate opioid-induced sociability deficits.

6.2 Methods

6.2.1 Experimental Subjects

Male and female Sprague Dawley rats (approximately two months of age) were used to generate offspring perinatally exposed to morphine or sterile saline (vehicle) for controls (Charles River Laboratories, Boston, MA). After weaning on P21, all rats were housed in Optirat GenII ventilated cages (Animal Care Systems, Centennial, Colorado, USA) in same-sex pairs with corncob bedding. Food (Lab Diet 5001 or Lab Diet 5015 for breeding pairs, St. Louis, MO, USA) and water were provided ad libitum throughout the experiment, except during testing. All studies were approved by the Institutional Animal Care and Use Committee at Georgia State University and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to reduce the number of rats used in these studies and minimize pain and suffering.

6.2.2 Perinatal Opioid Exposure Paradigm

Adult female Sprague Dawley rats (P60) were implanted with iPrecio SMP-200 microinfusion minipumps; morphine administration was initiated one week later. Pumps were programmed to deliver 10 mg/kg of morphine three times a day, with doses increasing weekly

by 2 mg/kg until 16 mg/kg was reached (Figure 1).

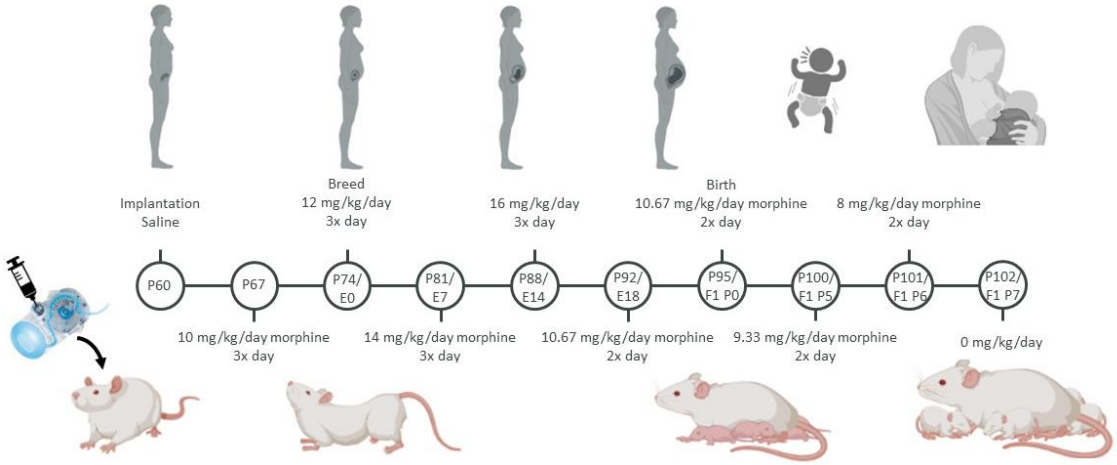

Figure B.1. Schematic of the perinatal opioid exposure dosing paradigm. Created with Biorender.com.

One week after morphine initiation, females were paired with sexually-experienced males for two weeks to induce pregnancy. Morphine exposure to the dams continued throughout gestation. At E18, approximately three days before parturition, pumps switched to 2x/day dosing, as initial studies using 3x/day dosing led to increased pup mortality following birth. Dams continued to receive morphine after parturition, such that pups received morphine through their mother's milk. Beginning at P5, morphine dosage was decreased by 2 mg/kg daily until P7, when the dams received 0 mg/kg morphine (i.e., sterile saline). This protocol closely mirrors the clinical profile of infants exposed to opioids *in utero*, in which supplemental opioid dosing following birth is used to minimize withdrawal (Kocherlakota, 2014). Vehicle rats were treated in an identical manner, except pumps were filled with sterile saline. Doses of morphine used in this study (10-16 mg/kg) are higher than the median effective dose [ED50] to induce analgesia to a thermal stimulus in female rats (Doyle et al., 2017), indicating that this dosing paradigm is representative of doses used in humans for pain relief.

6.2.3 Litter Characteristics and Dam/Pup Weight

Dams were weighed weekly during pregnancy to compare growth and weight gain between vehicle- and morphine-treated dams. At birth, litter size and sex balance were compared between vehicle- and morphine-exposed litters. At P4, anogenital distance (AGD) was measured using electronic calipers as a measure of potential endocrine disruption and/or differential androgen exposure (Schwartz et al., 2019). Pups were weighed every two days from P0 to P14, as neonatal morphine is associated with decreased growth and/or delayed developmental milestones (Najam and Panksepp, 1989).

6.2.4 Maternal Behavior

Maternal behavior was observed in the home cage during the light phase for one hour twice daily on P2, P4, and P6 to capture behavior during both high and low circulating levels of morphine. Following morphine cessation, maternal behavior was observed once daily on P8, P10, P12, and P14. Behaviors scored include nursing, licking/grooming, and time spent off pups.

6.2.5 Social Play Paradigm

The typical developmental arc of juvenile social play is an inverted U-shape centered around P35 (Panksepp, 1981; Veenema et al., 2013); therefore, social play was measured three times over adolescence (P25, P35, and P45) to allow for the identification of any potential developmental shifts in morphine-treated offspring. Rats were separated from their cagemate for 24 hours before each trial to facilitate social play (Niesink and Van Ree, 1989; Vanderschuren et al., 1995d). Social behavior was videotaped for offline analysis for 12 minutes under red light. Social play was scored by two observers blind to condition. Five metrics were scored:

1. Time spent engaged in social play: total time spent chasing, pinning, and performing nape attacks.
2. Time spent in social interaction: total time spent sniffing or grooming their partner's body.
3. Time spent not-in-contact: total time spent not interacting with each other (i.e., exploring the cage or walking around).
4. The number of pins: one rat lying on its dorsal surface with the other rat above it.
5. The number of nape attacks: one rat noses/rubs the neck of the other rat.

Behaviors 1-3 were scored per treatment- and sex-matched pair, while 4-5 were scored individually for each rat.

6.2.6 Stress Assessment

To assess separation-induced stress, rats were provided nestlet squares in their isolation cages. The nestlet square was pre-weighed, and the total percentage shredded was measured after twenty-four hours. Higher percentages of shredded material represent higher anxiety and/or compulsive behaviors resulting from isolation (Angoa-Perez et al., 2013). Previous studies have reported nestlet shredding is sensitive to anxiolytic drug administration, suggesting this is a valid indicator of anxiety-like behavior in rodents (Li et al., 2006).

6.2.7 Oxytocin Immunohistochemistry

The effect of perinatal opioid exposure (POE) on oxytocin peptide expression was assessed using immunohistochemistry. Male and female morphine- and vehicle-exposed rats (P7, P14, or P30) were decapitated, brains rapidly removed, and drop-fixed in 4% paraformaldehyde for 24 hours, followed by 30% sucrose until sectioning. Fixed tissue was

sectioned in a 1:6 series of 40-µm coronal sections with a Leica SM2010R microtome and stored in cryoprotectant at –20 °C. To visualize OT expression, free-floating sections were rinsed thoroughly in potassium phosphate buffer solution (KPBS), incubated in 3% hydrogen peroxide at room temperature for 30 minutes, and then rinsed in KPBS. Sections were then incubated in 1:10,000 mouse anti-oxytocin primary antibody (MAB5296, Millipore-Sigma) diluted in KPBS with 1% Triton-X overnight at room temperature. Following rinses in KPBS, sections were incubated in 1:600 biotinylated donkey anti-mouse secondary antibody (715-065-151, Jackson Immuno) diluted in KPBS with 0.4% Triton-X for one hour at room temperature. Following KPBS rinses, sections were incubated in an Avidin/Biotin solution (PK-6100, Vector Labs) for one hour at room temperature, followed by rinses in KPBS and sodium acetate. The sections were then incubated in a 3,3'-diaminobenzidine solution for 30 minutes, rinsed with sodium acetate and KPBS, and mounted onto slides. Slides were dehydrated using increasing concentrations of ethanol and cover-slipped. Oxytocin protein expression was quantified in the hypothalamic periventricular nucleus (PVN) and the supraoptic nucleus (SON), the main regions of oxytocin production and innervation (P7: 1-1.4 mm posterior to bregma; P14: 1.2-1.6 mm posterior to bregma; P30: 1.5-2.0 mm posterior to bregma). Four-six sections were analyzed, and the number of oxytocin neurons was counted and averaged per area for each rat.

Cresyl violet staining was performed on a subset of rats to investigate potential differences in overall cell number. Two-three 40-µm coronal sections per rat were mounted on slides and dipped in alternating solutions of 100% ethanol, xylene, 70% ethanol, 20% ethanol, water, cresyl violet/acetic acid solution, and differentiation solution (ethanol/acetic acid). Following cresyl violet staining, slides were cover-slipped and imaged for cell count analysis.

Total cell counts were calculated using ImageJ across the hypothalamus by an experimenter blinded to condition.

6.2.8 Statistical Analysis

Significant main effects of sex, treatment, age, and interaction effects were assessed using two- or three-way mixed models or repeated measures mixed models; $p < 0.05$ was considered significant. As repeated measures ANOVA cannot handle missing values, we analyzed our data by fitting mixed models with Greenhouse-Geisser correction (when the requirement of sphericity was not met) as implemented in GraphPad Prism 9.1.0 (Motulsky, 2021). Tukey's or Sidak's post hoc tests were conducted to determine significant mean differences between a priori specified groups. Due to the method of partitioning variance in linear mixed models, there is no universal method to calculate standardized effect sizes (e.g., $\eta 2$ for ANOVA). Whenever possible, we report unstandardized effect sizes, which agree with recommendations for effect size reporting (Pek and Flora, 2018), including the guidance of the American Psychological Association Task Force on Statistical Inference (Wilkinson, 1999). Outlier points were defined as greater than two standard deviations from the mean of the treatment/sex/age group and were removed from future analysis. All experiments included both male and female offspring to investigate possible sex-specific effects of POE on social behavior. No differences were observed between rats of different litters in the same drug exposure group (i.e., morphine-exposed and vehicle-exposed); therefore, rats from multiple litters [4 morphine-exposed litters; 5 vehicle-exposed litters] spanning 12 months, served as a single sample cohort. A range of 6-12 pups were used per litter.

6.3 Results

6.3.1 Litter Characteristics and Dam/Pup Weight

To determine the effects of morphine exposure on developmental milestones, we investigated the weight gain of dams and pups, along with litter size, sex balance, and anogenital distance. All dams gained weight equally throughout pregnancy, regardless of treatment [Time*Txt effect; $F(5,33)=1.742$, $p=0.1525$]. No significant differences in body weight of vehicle- and morphine-treated dams were detected from weeks 0-5 post surgery [Sidak post hoc, $p=0.5540$-0.9999]. Vehicle-treated dams weighed, on average, 11.43 grams more than morphine-treated dams, approximately 5% difference in body weight (Figure 2A). At birth, litter size was significantly smaller for morphine-exposed dams [$t(7)=2.38$, $p=0.0489$] (Figure 2B). Vehicle-exposed litters consisted of, on average, 12 pups, while morphine-exposed litters consisted of an average of 9 pups (25.8% fewer). No effect of morphine on sex balance was observed [$t(7)=0.48408$, $p=0.6453$] (Figure 2C). On average, vehicle-exposed litters were comprised of 61.8% male pups, while morphine-exposed litters were 54.8% male.

There were no sex differences in pup weight between P2-P21; therefore, the sexes were collapsed. Analysis of pup weight identified a significant interaction between age and treatment [$F(7,609)=6.443$, $p<0.001$]. While no significant differences in pup body weight were observed from P2-P21 [Sidak post hoc; $p=0.0910$-0.9], minor mean differences were observed across groups. Morphine-exposed pups weighed approximately 7% less than vehicle-exposed pups from P2-P10 but were 6% heavier than vehicle-exposed pups at weaning (Figure 2D). Anogenital distance (AGD), measured on P4, was not significantly different between sex-matched vehicle- and morphine-exposed pups [Txt effect, $F(1,84)=0.5853$, $p=0.4464$] (Figure

2E). Average AGD for vehicle- and morphine-exposed males was 4.37 mm. Similarly, vehicle- and morphine-exposed females had an average AGD of 2.2 mm. Together, these results indicate no differences in developmental milestones between morphine- and vehicle-treated dams and their offspring.

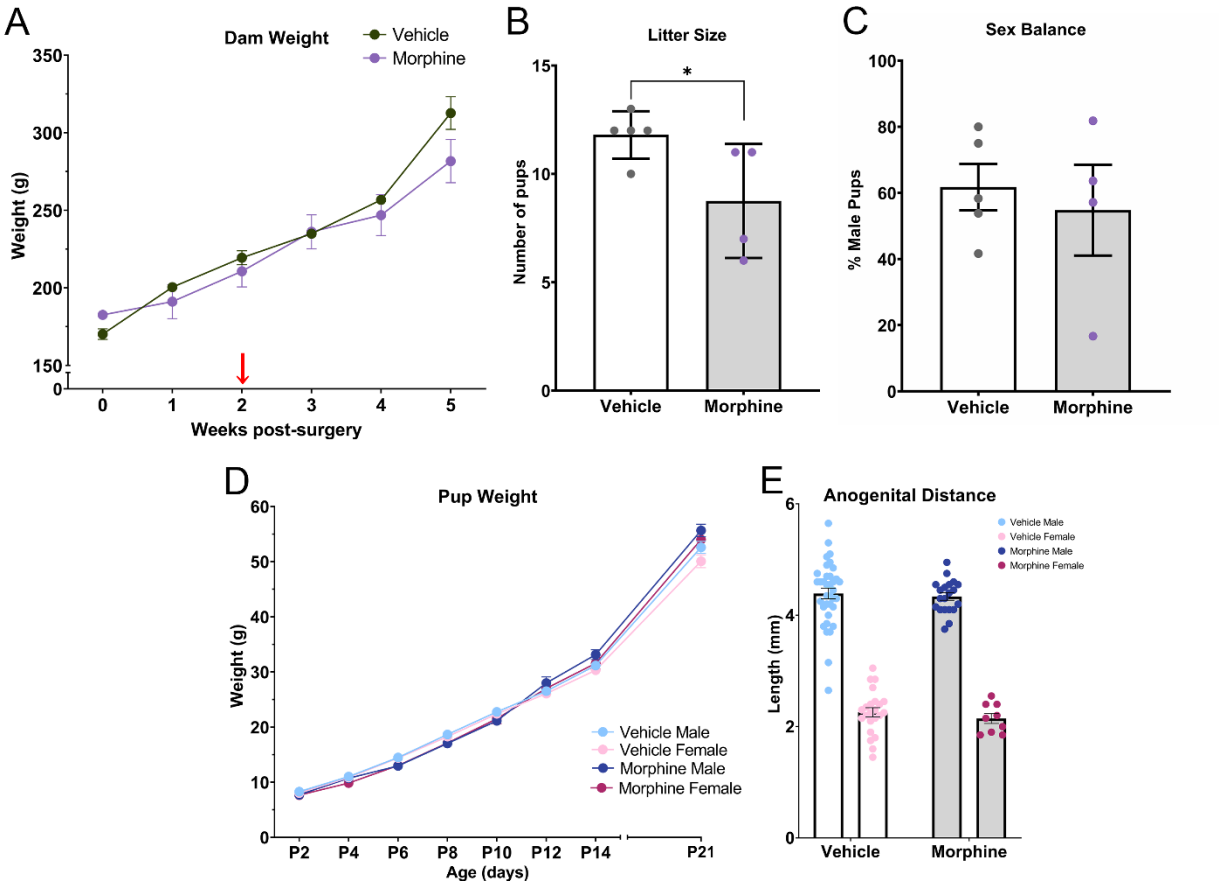

Figure B. 2. Perinatal opioid exposure does not lead to overt differences in litter characteristics or body weight. (**A**) Dams treated with morphine tend to weigh less later in pregnancy. Red arrow represents initiation of breeding. (**B**) Litter size was significantly reduced in morphine-treated dams. (**C**) No difference in sex balance between vehicle- and morphine-treated dams. (**D**) Male and female offspring exposed to morphine weigh less than vehicle-exposed offspring from P2-P10 but weigh more than vehicle-exposed offspring at weaning. (**E**) No effect of perinatal opioid exposure on anogenital distance. $N_{VEH\ M}$ = 36-43, $N_{VEH\ F}$ = 23-35, $N_{MOR\ M}$ = 16-20, $N_{MOR\ F}$ = 9-19. Graphs represent mean ± SEM. * = significant at $p < 0.05$.

6.3.2 Maternal Behavior

To examine the impact of morphine treatment on maternal behavior, we observed nursing and licking/grooming in the home cage of vehicle- and morphine-treated dams. Our analysis identified significant interactions of time and treatment for nursing [$F(9,72)=3.551$, $p=0.0011$] and licking and grooming [$F(9,72)=2.648$, $p=0.0104$]. Although individual comparisons were not significant

[Sidak post hoc; p=0.6187-0.9999], morphine-treated dams showed short-term decreases in nursing (30.1% less than vehicle-treated dams) when circulating levels of morphine were high (P2, P4, and P6 PM periods), but these differences were not observed at lower morphine levels (P2, P4, and P6 AM periods) or when morphine was no longer administered (after P8) (Figure 3A). Similar effects were observed with licking/grooming behavior (11.5% more than vehicle-treated dams) (Figure 3B). This effect on grooming was particularly noticeable at P6 PM [Sidak post hoc; p=0.0091], with morphine-treated dams grooming 15.7% more than vehicle-exposed dams. No other timepoints reached statistical significance [Sidak post hoc; p=0.7178-0.9999]. Overall, these data show that while morphine does impact maternal behavior, it has opposing effects on nursing (negative) and grooming (positive), and these effects are corrected once morphine exposure has ceased.

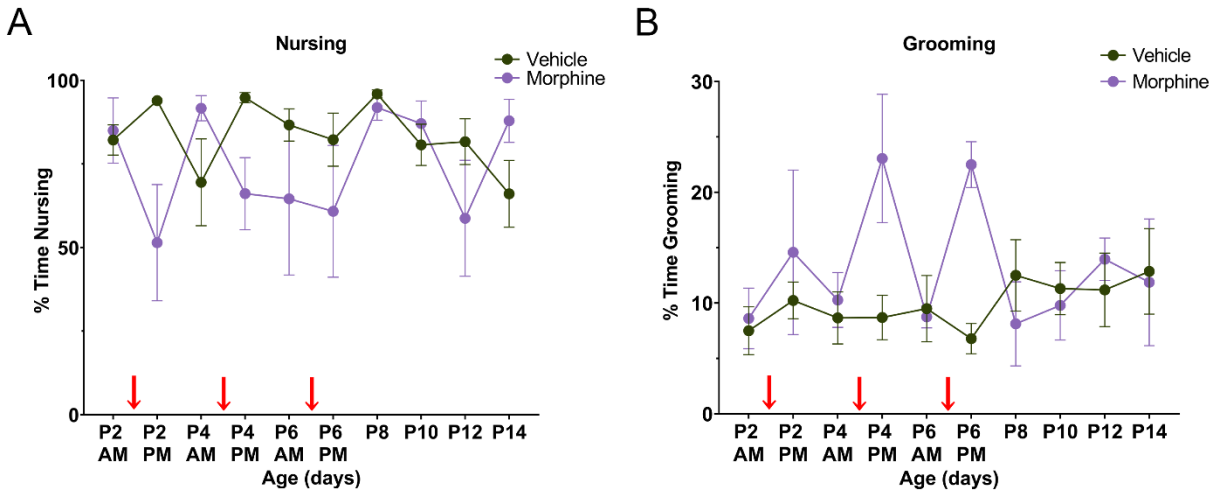

Figure B. 3. Maternal morphine treatment leads to short-term differences in maternal behavior. Dams treated with morphine spent less time nursing (**A**), and more time grooming (**B**) when morphine was administered (P2 PM, P4 PM, and P6 PM; indicated by red arrows). N_{VEH} = 7, N_{MOR} = 4. Graphs represent mean ± SEM. Red arrows indicate timing of morphine dose.

6.3.3 Adolescent Pup Weights

To investigate the potential long-term effects of POE on body weight and its relationship with play behavior, we measured weight at P25, P35, and P45. There were no differences in weight between vehicle- and morphine-exposed offspring at any age [Age*Txt effect; $F(2,167)=2.904$, $p=0.0576$] (Figure 4A), suggesting that POE does not lead to long-term differences in body weight or growth rate, as is sometimes observed with smaller litters (Parra-Vargas et al., 2020).

6.3.4 Nestlet Shredding

Social isolation is a known stressor for rats; therefore, we used nestlet shredding to determine if repeated isolation prior to juvenile play was equally distressing for vehicle- and morphine-exposed offspring (Angoa-Perez et al., 2013). Higher percentages of shredded material are associated with higher anxiety and/or compulsive behaviors resulting from

isolation (Angoa-Perez et al., 2013). Here, we found nestlet shredding increased as a function of repeated isolation [Age effect; $F(1.968,161.4)=86.24$, $p<0.0001$]. This increase was comparable between vehicle- and morphine-exposed offspring [Age*Txt effect; $F(2,168)=2.497$, $p=0.0854$] (Figure 4B). Although not significantly different [Tukey's post hoc; P25 VEH F vs MOR F, $p=0.9999$; P35 VEH F vs MOR F, $p=0.9987$; P45 VEH F vs MOR F, $p=0.793$], morphine-exposed females shredded the most at all three timepoints, with an average percent shredded of 65%, 10-20% higher than all other groups. In contrast, morphine-exposed males shredded the least of all four groups, with an average percent shredded of 48%, approximately 5% lower than vehicle-exposed males. This suggests that morphine-exposed females may find repeated isolation more stressful than the other treatment-sex groups.

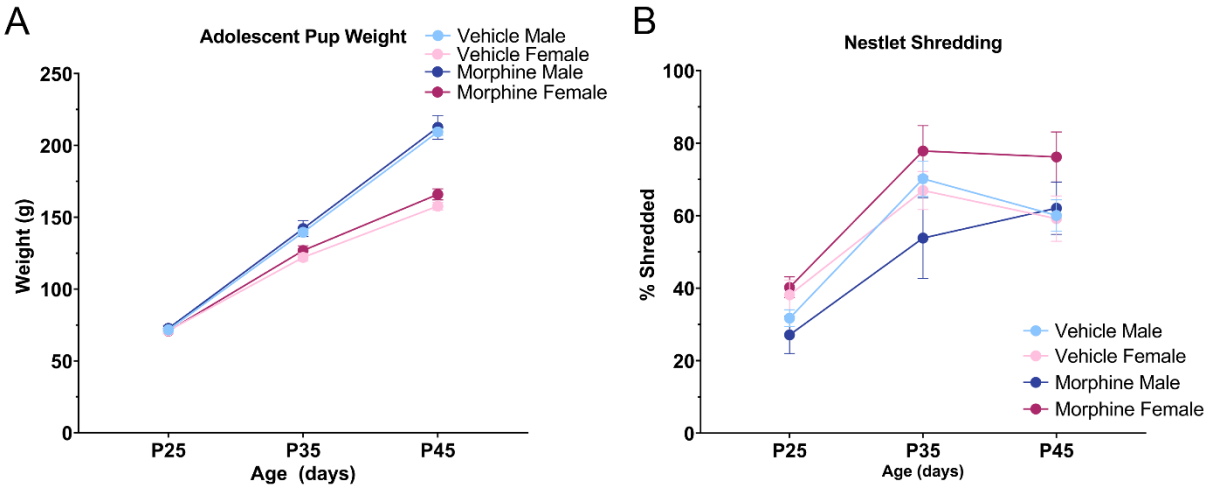

Figure B. 4. Adolescent weight (**A**) and nestlet shredding (**B**) do not differ between morphine- and vehicle-exposed male and female offspring. $N_{VEH\ M} = 35$, $N_{VEH\ F} = 25$, $N_{MOR\ M} = 12$, $N_{MOR\ F} = 17$. Graphs represent mean ± SEM.

6.3.5 Juvenile Play and Sociability

To determine the impact of perinatal morphine on juvenile social play, we next investigated five metrics of play: total time spent playing, time spent not-in-contact, time spent

in social interaction, and the number of nape attacks and pins performed by each rat. Analysis of social play identified a three-way interaction of age, treatment, and sex [F(2,57)=3.299, p=0.0441]. Although individual post hoc comparisons were not significant [Tukey's post hoc; P35 VEH F vs MOR F, p=0.9978; P45 VEH F vs MOR F, p=0.7359], at P35 and P45, morphine-exposed females had the lowest overall play times across all groups (Figure 5A). When averaged across all three developmental ages, morphine-exposed females spent the least time engaged in social play, although no significant effects of treatment or sex were observed [Txt*Sex interaction; F(1,24)=0.4183, p=0.5239] (Figure 5B). Specifically, morphine-exposed females played an average of 93.5 seconds in a 12-minute trial, 19.0% less than vehicle-exposed females, although this comparison did not reach statistical significance [Tukey's post hoc; p=0.8487]. No differences were noted in males, with morphine-exposed males playing an average of 135.5 seconds in a 12-minute trial, only 1.5% more than vehicle-exposed males. No sex difference in time spent not in contact was observed, so data were collapsed for analysis to increase power. ANOVA identified a significant effect of treatment [F(1,35)=4.164, p=0.0489] (Figure 5C), although no post hoc comparisons reached statistical significance [Sidak post hoc; P25 VEH vs MOR, p=0.6122; P35 VEH vs MOR, p=0.6068; P45 VEH vs MOR, p=0.2280]. Morphine-exposed females spent the greatest amount of time not-in-contact across all three timepoints, with an average of 82.6 seconds, a 54.5% increase vs. vehicle-exposed females [Tukey's post hoc; p=0.1393] (Figure 5D). Morphine-exposed males had the second highest not-in-contact time, with an average of 57.6 seconds, or a 30.4% increase vs. vehicle-exposed males [Tukey's post hoc; p=0.6985].

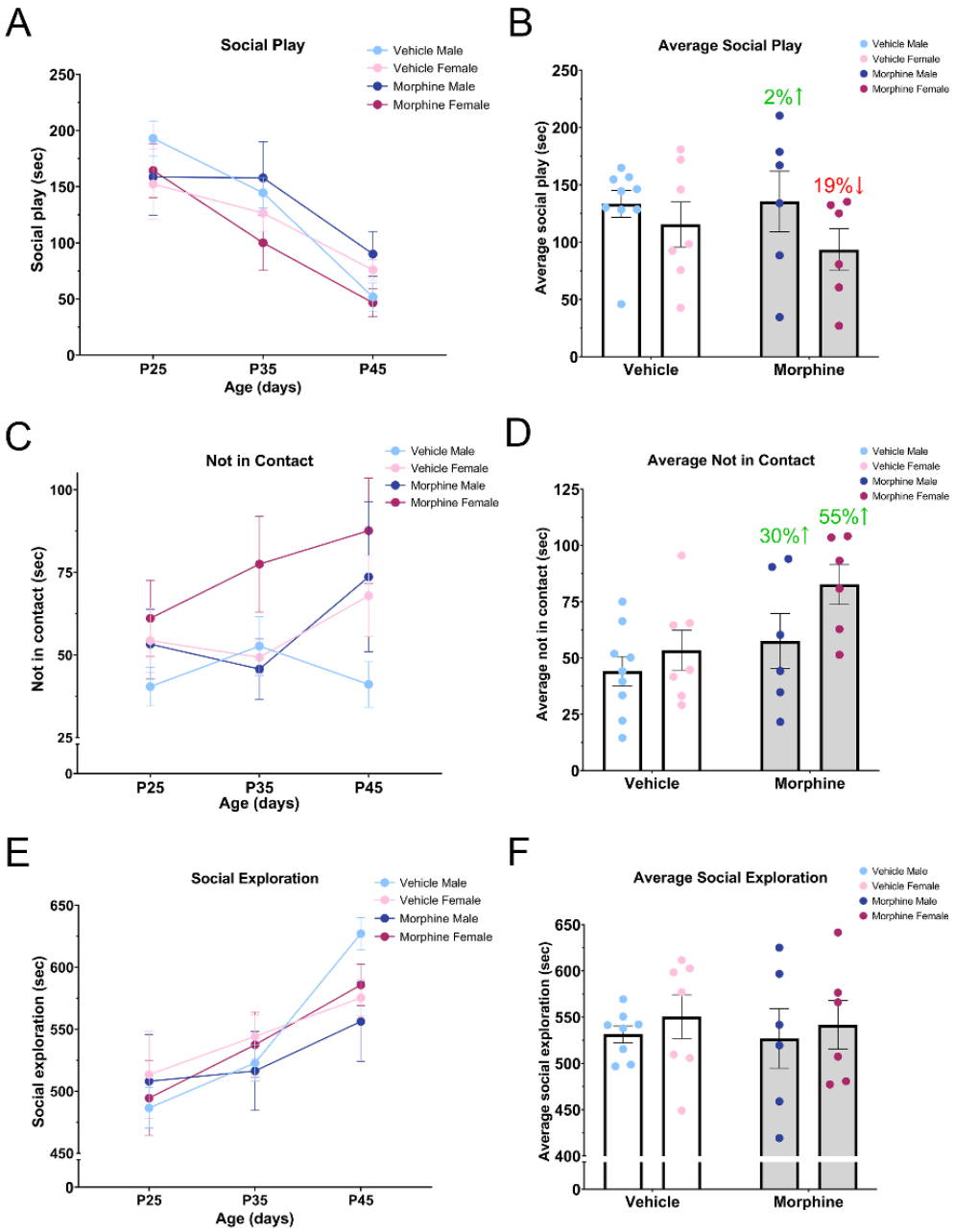

Figure B. 5. Morphine-exposed males and females show deficits in social behavior. (**A**) Morphine-exposed females play less than morphine-exposed males and vehicle-exposed male and females at P35 and P45 and (**B**) when averaged across all three ages. (**C**) Not-in-contact time was highest in morphine-exposed rats, regardless of sex and (**D**) when averaged across all three ages. (**E**) Vehicle-exposed males participate more in social exploration at P45, but there was no effect of treatment on average social exploration across all three ages (**F**). $N_{VEH\ M}$ = 12 pairs, $N_{VEH\ F}$ = 11 pairs, $N_{MOR\ M}$ = 6 pairs, $N_{MOR\ F}$ = 8 pairs. Graphs represent mean ± SEM. % represents change from sex-matched vehicle-exposed group.

To measure aspects of sociability independent from play, we assessed social exploration (licking, sniffing, and/or grooming the other rat). Analysis of social exploration identified a significant three-way interaction of age, treatment, and sex [$F(2,57)=3.296$, $p=0.0442$]. While social exploration time was approximately equal at P25 and P35, at P45, vehicle-exposed males spent 70.8 more seconds engaged in social exploration vs. morphine-exposed males, although this effect was not significant [Tukey's post hoc; $p=0.6688$] (Figure 5E). Females, regardless of treatment, showed comparable levels of social exploration at all three ages. When we analyzed average time spent across the three developmental timepoints, all groups showed comparable social exploration time [Txt*Sex effect; $F(1,23)=0.00892$, $p=0.9256$], with a range of 526-550 seconds (Figure 5F). Together, these data indicate that morphine-exposed rats are less engaged in social play, with a corresponding increase in not-in-contact time, and no corresponding alterations in social exploration.

6.3.6 Nape Attacks and Pins

We next examined nape attacks and pins, two characteristic features of rat juvenile play. We began with nape attacks, which often serve to initiate a play bout and occur when a rat noses the other rat's neck region. Analysis of nape attacks identified no treatment or sex differences; however, there was a significant effect of age [$F(2,125)=17.30$, $p<0.0001$], such that the number of nape attacks made at P35 and P45 was significantly lower than P25, regardless of treatment and sex group [P25 vs. P35, $p=0.0007$; P25 vs. P45, $p<0.0001$]. Although there was no significant effect of treatment [$F(1,72)=2.491$, $p=0.1189$], morphine-exposed females had the lowest number of nape attacks at P35 and P45 [Tukey's post hoc; P35 VEH F vs MOR F, $p=0.9935$; P45 VEH F vs MOR F, $p=0.9981$] (Figure 6A) and across all three ages, with an average

of 10.4 nape attacks, a 28.6% decrease vs. vehicle-exposed females [Tukey's post hoc; VEH F vs MOR F, p=0.5643] (Figure 6B). No difference in the average number of nape attacks was observed for vehicle- and morphine-exposed males [Tukey's post hoc; VEH M vs MOR M, p=0.999].

To compare the number of nape attacks made by each member of treatment- and sex-matched pairs, we calculated a nape attack difference score: the absolute value of the difference in the number of nape attacks each rat made. Pairs with similar numbers of nape attacks have low difference scores, while disparate numbers create high difference scores. Asymmetry in the number of playful initiations is a hallmark of well-developed dominant-subordinate relationships between cagemates and is most common among adult male rats (Pellis et al., 1997). All treatment and sex groups had lower nape attack difference scores as a function of age [F(1.995,50.87)=2.129, p=0.0336], and morphine-exposed male and female rats had lower nape attack difference scores overall [F(1,27)=4.316, p=0.0474] (Figure 6C), which reached significance at P25 [Sidak post hoc; P25 VEH vs MOR, p=0.0439], but not P35 [Sidak post hoc; P35 VEH vs MOR, p=0.4548] or P45 [Sidak post hoc; P45 VEH vs MOR, p=0.9845]. This effect was particularly noticeable in morphine-exposed females, with an average difference score of 3.6, 60.6% lower than vehicle-exposed females. Morphine-exposed males showed a less extreme reduction in nape attack difference score, with an average score of 8.8, or 18.3% lower than vehicle-exposed males.

We next examined pins, which often serve to complete a play bout and occur when one rat is laying on its dorsal surface while the other rat hovers above. Analysis of pins identified a significant interaction of age and sex [F(2,124)=4.184, p=0.0175] as well as an overall effect of

treatment [F(1,70)=4.647, p=0.0346] (Figure 6D). Morphine-exposed females made significantly less pins at P35 [Tukey post hoc; P35 VEH F vs MOR F, p=0.0043] and across all three ages, although this difference was not significant [Tukey's post hoc; VEH F vs MOR F, p=0.573]. However, mean differences were observed, with morphine-exposed females making an average of 7.8 pins, or a 25.8% decrease vs. vehicle-exposed females (Figure 6E). Morphine-exposed males made 10.7% fewer pins vs. vehicle-exposed males.

The difference score for pins was calculated similarly to the nape attack difference score, such that pairs of rats with similar numbers of pins have low difference scores, and pairs with disparate numbers of pins have high difference scores. Analysis of pins difference scores identified significant effects of age [F(1.431, 37.92)=8.546, p=0.0024] and treatment [F(1,27]=4.493, p=0.0434]. Treatment effects were particularly noticeable at P35 [Sidak post hoc, P35 VEH vs MOR, p=0.0449]. Morphine-exposed rats, regardless of sex or age, had a lower pins difference score [t(90)=2.435, p=0.0169]. This was especially noticeable in morphine-exposed females, who had the lowest difference score at P25, P35, and P45, although this difference did not reach statistical significance [Tukey's post hoc; P25 VEH F vs MOR F, p= 0.9483; P35 VEH F vs MOR F, p=0.2214; P45 VEH F vs MOR F, p=0.6121]. On average, morphine-exposed females had pins difference scores 27.2% lower than vehicle-exposed females; morphine-exposed males also showed a 14.7% reduction in pins difference score vs. vehicle-exposed males (Figure 6F). Together, these data suggest that morphine-exposed rats display fewer characteristic features of juvenile play and that perinatal morphine disrupts the formation of normal cagemate relationships, as indicated by reduced dominant-subordinate hierarchies (see Table 1 for summary of results).

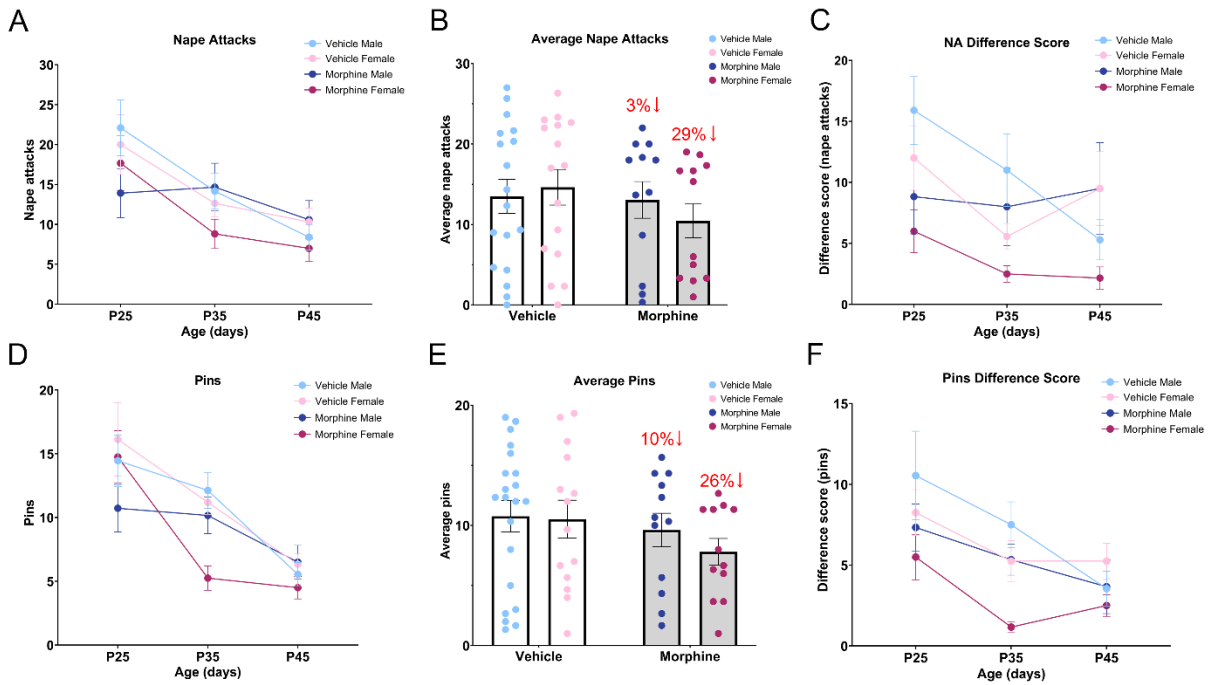

Figure B. 6. Morphine-exposed male and females display fewer characteristic features of social play. (**A**) Morphine-exposed females make the fewest nape attacks at P35 and P45 and (**B**) when averaged across all three ages. (**C**) Morphine-exposed males and females have significantly lower nape attack difference scores vs. vehicle-exposed males and females. (**D**) Morphine-exposed females make the fewest pins at P35 and P45 and (**E**) when averaged across all three ages. (**F**) Morphine-exposed males and females have significantly lower pin difference scores vs. vehicle-exposed males and females, particularly morphine-exposed females. Nape Attacks and Pins: $N_{VEH\ M}$ = 24, $N_{VEH\ F}$ = 22, $N_{MOR\ M}$ =12, $N_{MOR\ F}$ = 16. Difference Scores: $N_{VEH\ M}$ = 11 pairs, $N_{VEH\ F}$ = 8 pairs, $N_{MOR\ M}$ = 6 pairs, $N_{MOR\ F}$ = 6 pairs. Graphs represent mean ± SEM. % represents change from sex-matched vehicle-exposed group.

6.3.7 Oxytocin Immunohistochemistry

Previous studies have strongly implicated oxytocin in regulating play and sociability. Therefore, we next examined the impact of perinatal opioid exposure on oxytocin expression using immunohistochemistry (Figure 7A). No significant sex differences were observed for the PVN or SON, so males and females were collapsed to increase power. Analysis of the total number of oxytocin-positive (OT+) cells in the PVN identified a significant interaction of age and treatment [F(2,53)=5.044, p=0.0099] (Figure 7B). While no post hoc comparisons reached statistical significance [Sidak post hoc; P7 VEH vs MOR, p=0.1505, P14 VEH vs MOR, p=0.5017;

P30 VEH vs MOR, 0.0968], mean differences were observed. Morphine-exposed rats had decreased OT+ cell count at P7, but an elevated OT+ cell count at P14 vs. vehicle-exposed rats. At P30, the number of OT+ cells were comparable between vehicle- and morphine-exposed rats (Table 2). Together, this suggests that perinatal morphine exposure alters OT+ cells in the PVN at P7 and P14, which is mostly normalized by P30.

We next investigated the number of oxytocin-positive cells in the SON. Analysis of the total number of OT+ cells identified a significant interaction of age and treatment [$F(2,47)=23.70$, $p<0.0001$] (Figure 7C). Morphine-exposed rats had decreased OT+ cell count at P7 [Sidak post hoc, $p=0.0001$] and a non-significant elevated OT+ cell count at P14 [Sidak post hoc, $p=0.2518$] vs. vehicle-exposed rats. This increase reached statistical significance at P30 [Sidak post hoc, $p<0.0001$] (Table 3).

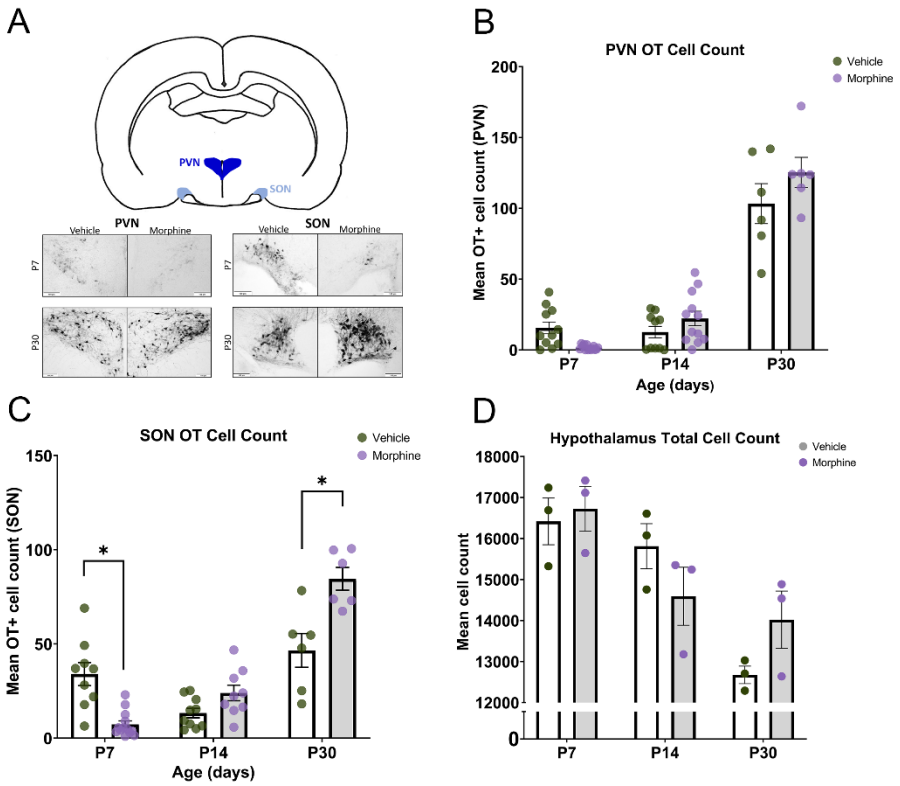

Figure B. 7. Morphine-exposed rats show decreased oxytocin expression in the PVN and SON. (A) Representative images of OT staining in the PVN and SON. (B) OT+ cell counts were reduced in morphine-exposed rats in the PVN at P7, increased at P14, and comparable at P30. (C) Similar results were observed in the SON at P7, P14, and P30. N_{VEH} = 6-11, N_{MOR} = 6-14. (D) Cresyl violet staining in the hypothalamus of a subgroup of rats showed no differences in overall cell count. N_{VEH} = 3, N_{MOR} = 3. Graphs (panels B-C) represent median ± IQR and (panel D) represent mean ± SEM. *: significant ($p<0.05$) Sidak post hoc analysis.

Importantly, these differences in OT+ cell counts in the PVN and SON are not due to differences in overall cell number in the hypothalamus [$F(2,12)=2.553$, $p=0.1192$] (Figure 7D). Cresyl violet staining revealed minor differences in total cell count for vehicle- and morphine-exposed rats with no effect of sex at P7, P14, and P30 [Sidak post hoc; P7 VEH vs MOR, $p=0.9774$; P14 VEH vs MOR, $p=0.5756$; P30 VEH vs MOR, $p=0.4616$]. Together, this suggests that perinatal morphine exposure leads to initial decreases in oxytocin cell counts, with a potential

compensatory increase in adolescence that is not due to overall differences in cell count in the hypothalamus.

6.4 Discussion

The present study was designed to investigate the impact of perinatal morphine exposure on juvenile play behavior and oxytocin peptide expression. We hypothesized that exposure to opioids during fetal development would lead to decreased oxytocin signaling, in line with morphine's known suppressive effects on oxytocin synthesis. Given the pro-social roles of oxytocin, we further hypothesized that morphine-induced changes in oxytocin signaling would negatively impact juvenile play. Our results showed a significant effect of treatment with reduced social play and altered oxytocin peptide expression. More specifically, males and females perinatally exposed to morphine engaged in less social play, particularly as they aged, as indicated by decreased time spent in social play and increased not-in-contact time. Although morphine-exposed females also showed increased isolation-induced stress behavior, this is not likely to explain their decreased social play, as isolation is typically associated with increased play (Panksepp, 1981). Morphine-exposed females did not display concurrent social exploration deficits in agreement with previous studies reporting that the impact of acute μ-opioid receptor agonists during the juvenile period is limited to play and not social exploration (Manduca et al., 2014; Trezza et al., 2011; Van Ree and Niesink, 1983). While analysis of social exploration in male rats did reveal non-significant mean differences in social exploration time, these differences are restricted to P45 and are not seen generally in morphine-exposed females or at other timepoints tested in this study. Our perinatal morphine administration paradigm recapitulates the clinical profile of human infants exposed to opioids *in utero*, incorporating

opioid exposure before and during gestation, and immediately after parturition. An essential part of this model is the inclusion of morphine before E15, the approximate date of μ-opioid receptor development in the fetal rat brain (Coyle and Pert, 1976). As there are potential trophic effects of opioids early in development (Kuhn et al., 1992), the use of a perinatal administration paradigm improves the clinical relevance and translatability of our findings to humans with *in utero* opioid exposure.

In the present study, lower OT+ cell count in the SON was observed at P7 in morphine-exposed rats, likely due to morphine's inhibitory effects on oxytocin synthesis. Morphine-exposed rats showed elevated OT+ cell count in the SON at P30, suggesting a potential "rebound" or compensatory increase. Although not directly tested in the present study, we speculate that decreases in oxytocin expression observed in this study are likely due to the presence of morphine during critical periods of brain development and contributed to the observed deficits in adolescent social play. Opioids, particularly μ- and κ-receptor agonists, generally inhibit OT and vasopressin (AVP) neuronal firing, thereby suppressing peptide release (Douglas and Russell, 2001; Inenaga et al., 1990; Li et al., 2001; Lutz-Bucher and Koch, 1980; Van de Heijning et al., 1991), likely via presynaptic mechanisms (Clarke and Wright, 1984; Zhao et al., 1988). Interestingly, juvenile play also facilitates the development of inhibitory prefrontal cortical synapses necessary for cognitive and executive function (Bijlsma et al., 2022). This suggests that clinical reports of reduced social behavior and deficits in cognitive performance may be inter-related (Yeoh et al., 2019).

Our results are consistent with previous studies reporting that chronic opioid administration to adult male rats significantly decreases OT in both the PVN and SON (Laorden

et al., 1997; Laorden et al., 1998; You et al., 2000). Together with the present results, this suggests that opioid-induced downregulation of oxytocin expression is not limited to specific developmental windows and is a likely mediator of opioid-induced changes in sociability. Importantly, people with opioid use disorder have higher rates of social isolation, which often cyclically produces greater levels of drug use and further feelings of social isolation (Christie et al., 2021). This was particularly prevalent during the COVID-19 pandemic, in which rates of opioid use and overdose significantly increased (Alter and Yeager, 2020; Niles et al., 2021). Opioid-induced decreases in oxytocin production may be one mechanism underlying this bidirectional, cyclic relationship between opioid exposure and social isolation. This connection may also suggest that children who were exposed to opioids *in utero* would be more susceptible to drug use and addiction later in life, as is seen in other preclinical models of gestational opioid exposure (Gagin et al., 1997; Ramsey et al., 1993; Shen et al., 2016; Torabi et al., 2017; Wu et al., 2009).

The results of the present study have implications for the clinical population of opioid-exposed infants that show sociability deficits, including social difficulties and inattention (Sandtorv et al., 2018). For example, children prenatally exposed to opioids were reported to have significantly higher scores on the “social difficulties” subscale of the Autism Spectrum Screening Questionnaire, including a lack of empathy and eye contact, no or few friends, and being poor at playing games (Sandtorv et al., 2018). These deficits are commonly observed in autism spectrum disorder and attention deficit hyperactivity disorder (Alessandri, 1992; Bredewold et al., 2014; Jordan, 2003; Reppucci et al., 2018), particularly in children. Alterations in OT signaling are implicated in social play deficits and ASD (Reppucci et al., 2018), including

lower OT levels and polymorphisms in the OXTR gene (Meyer-Lindenberg et al., 2011; Zhang et al., 2017). OT administration has been reported to improve social functioning in children with ASD (Meyer-Lindenberg et al., 2011), suggesting the OT system is a prime therapeutic target for improving social play deficits in children with *in utero* opioid exposure.

In summary, the current study provides evidence that perinatal morphine exposure disrupts early life oxytocin signaling in the social reward circuit, resulting in atypical adolescent social play. Specifically, we show that perinatal morphine decreases key features of social play and that this decrease is associated with a decrease in OT in regions implicated in social behavior and reward (see Figure 8 for a summary of proposed model). The use of a clinically relevant model of perinatal opioid exposure boosts the translational value of these studies in informing the development of potential clinical interventions for improving sociability in infants with *in utero* opioid exposure.

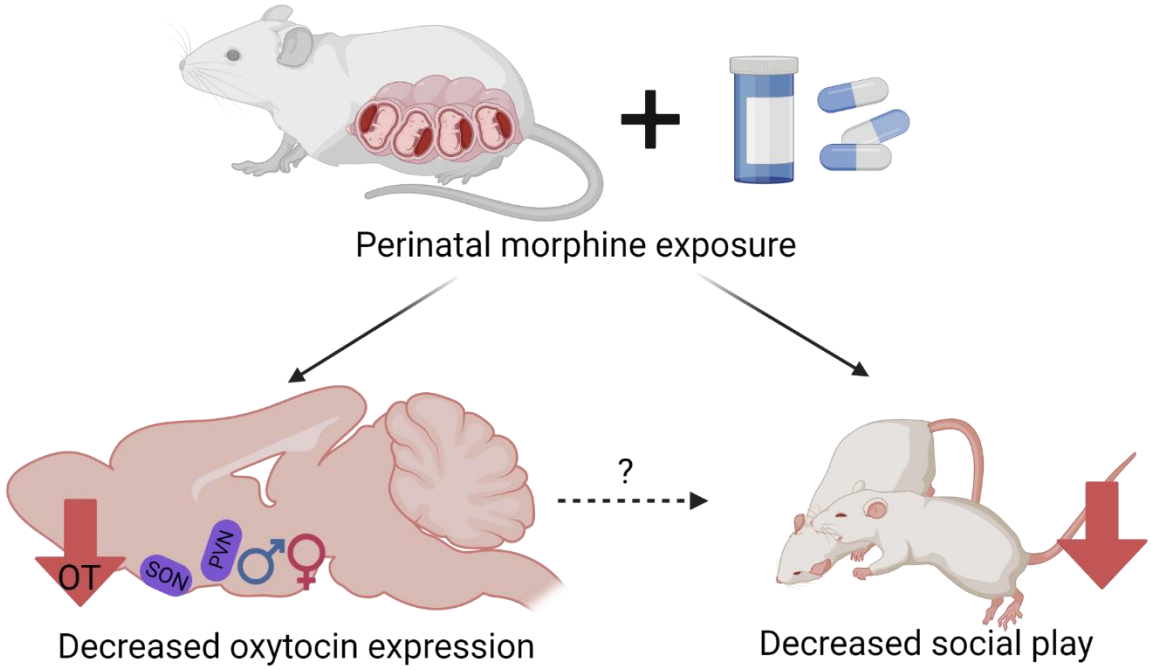

Figure B. 8. Model of proposed effect. Perinatal morphine exposure leads to decreased oxytocin expression in the PVN and SON in males and females. This suggests that perinatal morphine results in dysregulation of the oxytocin system during a critical period of social development, likely leading to long-term changes in juvenile play. Created with Biorender.com.

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	MOR
Nestlet Shredding	—
Social Play	↓
Not-in-Contact	↑
Social Exploration	↓
Nape Attacks	—
Nape Attack Difference Score	↓
Pins	↓
Pins Difference Score	↓

Table B. 1. Summary of behavioral results.

PVN	P7	P14	P30
VEH	15.6 ± 13.8	12.6 ± 12.7	103.3 ± 34.6
MOR	1.6 ± 1.6 (↓89%)	22.2 ± 17.9 (↑76%)	125.4 ± 25.9 (↑21%)

Table B. 2. OT+ cell counts in the PVN for vehicle- and morphine-exposed rats at P7, P14, and P30. % represents percent change of morphine-exposed vs. vehicle-exposed rats.

SON	P7	P14	P30
VEH	34.0 ± 8.4	13.3 ± 8.0	46.6 ± 22.0
MOR	7.3 ± 6.6* (↓79%)	24.0 ± 12.4 (↑80%)	84.6 ± 14.8* (↑80%)

Table B. 3. OT+ cell counts in the SON for vehicle- and morphine-exposed rats at P7, P14, and P30. *: significant ($p<0.05$) Sidak post hoc analysis. % represents percent change of morphine-exposed vs. vehicle-exposed rats.

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