MODELING MATERNAL OPIOID USE DISORDER AND ITS CONSEQUENCES IN MICE OFFSPRING

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“Education is the most powerful weapon which you can use to change the world.”

-Nelson Mandela

This thesis is dedicated to my family –

I will always be immensely grateful for all your sacrifices and for always instilling in me the power of having an education. Like my grandmother Ata would say – la educación es lo único que no te pueden quitar.

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ABSTRACT

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The opioid epidemic has seen an increase in drug use among women of reproductive age. Opioid Use Disorder can have many negative consequences for the health of pregnant mothers and their babies, but our understanding of the impact of fetal opioid exposure on behavior during adolescence and adulthood is less understood. Preclinical studies have unveiled some of the long-term effects of *in utero* morphine exposure primarily using injections, osmotic minipumps, and forced oral opioid drinking as the route of drug delivery. This dissertation presents a collection of experiments using a (i) prenatal-perinatal and (ii) prenatal-preweaning morphine exposure paradigm to describe the effects on neonate, adolescent, and adult offspring behavior. First, we validated a paradigm where female mice first became morphine dependent pre-pregnancy, then continued to consume morphine in a continuous two-bottle choice (2BC) paradigm during pregnancy and up to offspring postnatal day (PND) 7, at which point offspring were cross-fostered (prenatal-perinatal), or up to offspring PND 21 (prenatal-preweaning). We demonstrated that morphine dams display signs of dependence and voluntarily drink morphine throughout gestation.

Offspring exposed to prenatal-perinatal morphine displayed changes in neonate outcomes, including ultrasonic vocalization parameters. They also showed changes in anxiety-like behavior, ethanol intake before and after an acute stressor, and a greater percentage of them had more severe global behavioral scores. Next, we demonstrated that offspring exposed to morphine prenatally and up to the time of weaning display changes in adolescent behavior,

including alterations in preference for nicotine in a 2BC, transcriptomic changes in the Prefrontal Cortex, and an increased proportion of offspring classifying in the more severe behavioral phenotypes. During adulthood, the same offspring displayed differences in behavioral phenotype classification, and changes in ethanol intake and/or preference in an intermittent 2BC paradigm and a binge-like drinking procedure. Overall, prenatal-perinatal and prenatal-preweaning morphine exposure leads to subtle sex-specific changes in neonatal, adolescent, and adult behavior. Altogether, this body of work contributes to the current understanding of the effects of *in utero* opioid exposure and potential vulnerable/resilient populations among offspring.

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CHAPTER 1: A General Introduction

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Adapted from draft of a review written with: Katherine R. Webb and Mariella De Biasi

1.1. Epidemiological data on opioid epidemic and Opioid Use Disorder

The opioid epidemic, which first received attention during the Vietnam War when heroin use rose rapidly among U.S soldiers, has remained a pressing public health crisis in the United States (Hall & Weier, 2017; James & Jordan, 2018; Nixon, 1971). In fact, though rarely mentioned, minority communities faced an equally pressing opioid epidemic in the 1960's and 70's (James & Jordan, 2018). Opioid Use Disorder (OUD) is defined in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5) as a problematic pattern of opioid use that significantly impairs one's way of living and causes distress, including craving, tolerance, and withdrawal (Center for Disease Control, 2016). Incidence of OUD has risen nearly 6-fold in the last two decades, with an estimated 1.7 million people reporting misuse of opioids in 2017 (Fleming, 2018). This increase can be traced in large part to pharmaceutical companies who minimized the data on the abuse liability of opioids, which lead to overprescribing of oxycodone prescriptions, and a sharp increase in cases of opioid dependence (Dayer et al., 2019). Additionally, the advent of cheap, synthetic derivatives like fentanyl has led to an unprecedented number of opioid-related overdose deaths. In 2017, 47,600 Americans, equating to nearly 200 per day, died of an opioid-related overdose (Hedegaard et al., 2018).

Just within the past three years, there has been an increase in overdose deaths during the COVID-19 pandemic at the national, city, and regional level. Specifically, there was a 58% increase in overdose deaths from 2019 to 2020 (Friedman & Akre, 2021; Ghose et al., 2022; Patel et al., 2021). This new wave of overdose deaths is largely due to illicit fentanyl use (Ciccarone, 2021). In certain regions, opioid overdose has disproportionately affected low-income Black and Hispanic communities, when comparing opioid overdose death patterns before and after the pandemic (Ghose et al., 2022; Patel et al., 2021). Targeted- and evidence-based interventions are needed in every community, especially in underrepresented communities that have been historically neglected and lack access to proper medical care.

1.2. Morphine mechanism overview

I will specifically give an overview of morphine, given that it is the drug of focus for my dissertation.

Morphine, the active metabolite of heroin, is one of the natural alkaloids found in *papaver somniferum,* more commonly known as the opioid poppy*,* and the prototypical opioid against which all other opioid derivatives are measured (Brook et al., 2017; Rosenblum et al., 2008; Stefano et al., 2017). The naturally derived compounds from *P. somniferum* include: morphine, codeine, thebaine, and papaverine (Pathan & Williams, 2012). After the isolation of morphine, semi-synthetic compounds were derived, including heroin (diamorphine), dihydromorphone, buprenorphine, and oxycodone. Synthetic compounds were also made, and include fentanyl and methadone. Opioid

receptors are a family of G-protein coupled receptors that includes μ (MOR), δ (DOR), and κ (KOR) receptors. Each of the opioid receptor subtypes is comprised of seven transmembrane proteins that couple to the inhibitory G-proteins, G_i and G_o. Upon binding of opioid agonists, these receptors engage downstream intracellular signaling cascades that result in, among other effects, decreased cAMP production, inhibition of calcium ion channels and, ultimately, depression of neural activity, or hyperpolarization (Corder et al., 2018; Lamberts et al., 2018; Toll et al., 2016). A more in-depth review on the pharmacology of opioids can be found by Pathan and Williams (2012).

Opioids can be categorized based on whether they are an agonist, partial agonist, or antagonist at opioid receptors. Morphine, oxycodone, methadone, and fentanyl are opioid agonists, whereas buprenorphine is a partial agonist which elicits a partial response upon μ opioid receptor binding.

Morphine binds to μ, δ, and κ opioid receptors but it displays the highest affinity for the μ subtype of receptors (Corbett et al., 2006). In the clinical setting, morphine is used to produce analgesia and sedation but there are dose-dependent side effects, including respiratory depression, bradycardia, nausea and vomiting, and reduction of gastric motility (Pathan and Williams 2012). These effects often involve overlapping mechanisms and are reflective of the expression of opioid receptors both in the central nervous system and in peripheral organs like the heart and the gastrointestinal tract (Montandon & Slutsky, 2019).

Studies using μ-opioid receptor knockout mice have revealed that this particular receptor subtype is critical not only for mediating morphine-induced analgesia, but also for producing strong rewarding effects which can lead to addictive behavior (Gavériaux-

Ruff & Kieffer, 2002; Kieffer & Gavériaux-Ruff, 2002; Lutz & Kieffer, 2013; Matthes et al., 1996). Binding of morphine to opioid receptors located within the reward pathway [including the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC)] produces euphoria and other pleasurable feelings that can act as positive reinforcers (Al-Hasani & Bruchas, 2011).

The primary mechanism by which opioids exert their effects in the reward pathway is through disinhibition. Stimulation of inhibitory μ-opioid receptors on gamma-aminobutyric acid (GABA) interneurons in the VTA leads to decreased GABA release onto dopaminergic VTA neurons (Contarino et al., 2002; Dacher & Nugent, 2011; Fields & Margolis, 2015). This disinhibition of dopaminergic neurons results in greater dopamine release into the NAc, which ultimately facilitates the euphoric and reinforcing properties of morphine. More recently, studies have shown that opioids also act on μ-opioid receptors on other neurons within this circuitry, including GABAergic neurons in the rostromedial tegmental nucleus (RMTg) and feedback projections from the NAc, further enhancing dopamine release (Darcq & Kieffer, 2018). In contrast to stimulation of μ-opioid receptors within the VTA-NAc circuit, stimulation of kappa opioid receptors (KORs) is aversive and produces dysphoria. KORs located on dopaminergic neurons are a critical component of the brain's anti-reward system and produce dysphoria via presynaptic inhibition at both NAc and PFC terminals, among other mechanisms (Margolis et al., 2006; Tejeda et al., 2013).

Interestingly, neuroadaptations in both the reward and aversion circuits have been shown to contribute to the physical and psychological signs of dependence, including tolerance, withdrawal symptoms, craving, anticipation and obsessive thoughts regarding drug use (Darcq & Kieffer, 2018; T. R. Kosten & George, 2002; Langlois &

Nugent, 2017). Morphine tolerance, withdrawal, and the phenomenon of opioid-induced hyperalgesia have been widely reported in both clinical and preclinical studies (Kaneyasu, 2012; Moultrie et al., 2017; Pacifici, 2016; Rozisky et al., 2016; Zhang and Sweitzer, 2008; Zissen et al., 2007). In newborns, a retrospective review revealed that prolonged opioid administration (5 days or longer) was associated with tolerance, evidenced by infants requiring an increased number of drug infusions to maintain the same clinical effect, and withdrawal, evidenced by 13/23 infants displaying signs of physical discomfort after cessation of opioid infusions (Anand et al 2010). Interestingly in preclinical studies, neonatal mouse pups have also been found to display tolerance to morphine's analgesic effect (Barr and Wang, 1992; Fanselow and Cramer, 1988.) and display signs of emotional distress measured using ultrasonic vocalizations upon precipitated withdrawal (Barr & Wang, 1992).

1.3. Maternal opioid use and consequences to mother-child dyad, including Neonatal Opioid Withdrawal Syndrome (NOWS)

An important concern is the rise in OUD among women of reproductive age, leading to a greater incidence of adverse health outcomes for both pregnant women and their children (Azuine et al., 2019; Salihu et al., 2019). The current recommended treatment for pregnant women with OUD involves pharmacological interventions, known as maintenance therapies, with prescribed opioid agonists, and are given to newborns with severe NOWS as well (Patrick et al., 2017). These interventions are classified as forms of harm reduction. They serve to lower participation in drug-related crimes, to discourage the use of other illicit drugs, and to reduce the incidence of morbidity and mortality (Comer et al., 2015; Kreek et al., 2019; Stotts et al., 2009). Pharmacologically,

opioid maternal maintenance therapies help expecting mothers to maintain stable opioid blood levels, prevent withdrawal syndrome and reduce maternal craving for opioids (Hendrée E. Jones et al., 2012; Kaltenbach et al., 2018; Zedler et al., 2016). Importantly, maintenance therapies require a prescription from a licensed physician, bringing mothers into closer and more frequent contact with healthcare clinics, which increases the level of prenatal care and improves fetal and infant outcomes compared to mothers with untreated OUD or undergoing opioid withdrawal (Kelty & Hulse, 2017).

Despite the benefits of pharmacological interventions that reduce OUD in women, there is evidence that gestational exposure to opioids is a concern for the fetus because opioids can be transferred through the placenta and impact fetal development (Griffiths & Campbell, 2015; Malek & R. Mattison, 2011). *In utero* exposure to opioids has been linked to several negative outcomes that have been extensively reported on, including higher rates of spontaneous abortion and still birth, fetal growth retardation, changes in brain volume, white matter lesions, neonatal mortality and neonatal opioid withdrawal syndrome (NOWS) (Table 1-1) (Behnke & Smith, 2013; Broussard et al., 2011; Conradt et al., 2019; Tobon et al., 2019; Weller et al., 2020). Today, it is estimated that 5.8 in every 1000 newborn babies experience NOWS, a condition characterized by a variety of physiological and affective signs including loose stools, vomiting, temperature dysregulation, irritability, inability to be soothed, tremors, poor feeding, and high-pitched crying (Table 1-1) (Devlin & Davis, 2018; Patrick et al., 2017). An infant may experience NOWS regardless of whether the mother was using illicit opioids like heroin or taking opioids prescribed to treat her OUD. It has been reported that when pregnant opioid-dependent women are treated with methadone maintenance treatment (MMT), nearly 90% of their newborns will experience symptoms of NOWS, although the

severity of NOWS symptoms can vary significantly (Hendree E. Jones et al., 2005; Lejeune et al., 2006; Reddy et al., 2017). Merhar *et al*. (2019) reported that 40% of opioid-exposed newborns exhibit significant brain alterations such as punctate and diffuse white matter injuries, suggesting that the disruption of key processes during the development of the nervous system might increase vulnerability to behavioral and cognitive deficits later in life. Unfortunately, the long-term outcomes for children who are exposed to opioids, *in utero* and as neonates, and the mechanisms by which this experience enhances long-term vulnerabilities remain unclear.

Although not the focus of this introduction, methadone and buprenorphine are commonly prescribed drugs used as maintenance therapies for pregnant females with OUD. Many clinical studies have published data suggesting that, when treated with buprenorphine, newborns undergoing NOWS have shorter hospital stays and better outcomes than newborns treated with methadone or morphine (Hendrée E. Jones et al., 2010; Lemon et al., 2018). Interestingly, newborns treated with methadone have less severe NOWS symptoms and decreased hospital stays than those treated with morphine (Davis et al., 2018). Non-pharmacological approaches, like low environmental stimulation, breastfeeding, rooming in, swaddling, and skin-to-skin contact have also been shown to be important to lower NOWS severity and are being used as a first-response before giving the newborns pharmacological treatments (Hendrée E. Jones & Kraft, 2019; Piccotti et al., 2019). These clinical findings have also been backed by a growing number of preclinical studies which point to the effectiveness of alternative pharmacological and non-pharmacological treatments for NOWS. As with understanding the effects of prenatal morphine on offspring outcomes, preclinical studies will be critical

in determining both the short and long-term brain-level and behavioral effects of these treatments.

While retrospective clinical studies analyzing the immediate and long-term consequences of pre- and post-natal opioid exposure are abundant, they are also confounded by numerous factors, including discrepancies in self-reporting, co-use of other substances, undiagnosed mental health issues, and varying social and economic status, among others. For example, as many as 95% of pregnant women with OUD report tobacco use and up to 35% cite concurrent cannabis, cocaine and benzodiazepines use (Tobon et al., 2019). Narcotic recreational use is also associated with various underlying psychiatric conditions, notably depression and suicide, as well as malnutrition, anemia, and domestic violence (Arnaudo et al., 2017; Havens et al., 2009; Shantna et al., 2009). Each of these factors likely contributes to variations seen in the developmental and cognitive outcomes of newborns exposed to drugs *in utero*. Clinical studies, even randomized clinical trials (RCT's), are also complicated by the fact that pregnancy itself alters, and may exacerbate, inter-individual differences in the pharmacokinetic profiles of opioid agonists (Feghali et al., 2015; McLemore et al., 2013; G. H. Zhang & Sweitzer, 2008; H. Zhang et al., 2018). Moreover, there are very few examples of retrospective studies that have examined the long-term consequence of *in utero* opioid exposure, especially considering that underlying vulnerabilities might manifest several years after birth and might be masked by other conditions (Yeoh et al., 2019; Zedler et al., 2016).

1.4. Variability in clinical outcomes of children exposed to *in utero* opioids

As mentioned above, there are various types of opioids that pregnant women use, including methadone, buprenorphine, and morphine. Maternal drugs of recreational use, such as opioids, typically affect the systems in the fetal CNS that are developing at the time of drug exposure (Hauser & Knapp, 2018; Ross et al., 2015) which is reflected by the literature reporting apparently conflicting outcomes following *in utero* exposure to opioids. In addition, morphine is still considered the first line of treatment to reduce severe cases of NOWS symptoms in newborns. In one study, 50% (70/139) of newborns born from mothers on opioid maintenance therapies (OMT) were treated with morphine sulfate to treat NOWS (Welle-Strand et al., 2013). In humans, morphine readily crosses the placental barrier, leading to fetal-maternal plasma morphine ratios close to 1 (Gerdin et al., 1990). Such a high ratio likely reflects rapid transfer and equilibration of the drug between the mother and the fetus, with the clear potential to affect both placental and embryonic development (Collins et al., 2005; Garland et al., 2008; Ornoy et al., 1996).

There is conflicting evidence regarding outcomes associated with *in utero* opioid exposure. Infants exposed to *in utero* opioids have a higher risk of preterm birth and having to be transferred to the intensive care unit (Corsi et al., 2020). Several clinical studies have reported decreased body weight in newborns exhibiting NOWS symptoms (Siu & Robinson, 2014). However, other clinical studies report no growth differences (Corr et al., 2018; Kaltenbach et al., 2018). Another study showed that outcomes, such as head circumference and intellectual performance during pre-school, for opioid-exposed children did not differ from a control group after adjusting for covariates (Lifschitz et al., 1985). A systemic review and meta-analysis assessing ophthalmic outcomes in children exposed to *in utero* opioids also reported that there is not enough evidence to conclude a causal relationship, seeing that some studies report eye

abnormalities in newborns but some of these associations do not persist later in their lifespan (Hemmati et al., 2022).

Many reviews and meta-analysis of clinical data have reported infant/adolescent outcomes associated with prenatal opioid exposure, including lower scores in neurocognitive and developmental assessments, decreased motor skills, and increased hyperactivity and aggression (Maguire et al., 2016; Minnes et al., 2011; Nygaard et al., 2017; Weller et al., 2020; Yeoh et al., 2019). In addition, individuals who were exposed to opioids *in utero* may present with neurological and cognitive disturbances later in life (Minnes et al., 2011). The duration and amount of prenatal opioid exposure can differentially affect certain aspects of infant development, like motor skills and communication, which can vary at the age of analysis (Labella et al., 2021). One longitudinal study showed that a significantly higher proportion of youths whose mothers used heroin, among other drugs during pregnancy, had experienced a major depressive episode, alcohol abuse, Attention Deficit Hyperactivity Disorder (ADHD), and had more sexual partners during their lives (Nygaard et al., 2020). A proportion of those exposed to *in utero* opioids are also likely to experience traumatic events during childhood and/or adolescence that may be related to unfavorable socioeconomic conditions (Afful et al., 2010; Wells, 2009).

Interestingly, some studies suggest that it is highly variable how vulnerable an individual that was exposed to prenatal opioids is (Labella et al., 2021; Sarfi et al., 2021). Important factors that influence and can exascerbate human offspring outcomes include: mother's poly-drug use, socioeconomic stress experienced by pregnant mothers, and stressors associated with offspring being raised in foster/adoptive homes. In fact, a study comparing the mental health status of children of mothers who were on OMT compared

to children in foster-placement revealed a wide range of outcomes. The data suggest that there are subgroups in children from OMT-exposed mothers that might be more at-risk and warrant closer investigation and/or intervention (Sarfi et al., 2021). For instance, the OMT foster care group and control foster care group had higher rates of mental health problems compared to the OMT home group living at home at the time of the assessment (Sarfi et al., 2021). One study reported that only ~8% of children whose mothers used heroin still lived with their birth parents by three years of age (Lifschitz et al., 1985), suggesting that following these children in their various home environments will be important to further understand how this impacts outcomes.

Considering these confounds, preclinical studies offer a distinct advantage in identifying the factors that confer vulnerability to develop severe NOWS and the potential life-long consequences of *in utero* opioid exposure. This is the focus of Chapters 2-4 of this dissertation, which investigates how two distinct maternal opioid exposures (prenatal-perinatal and prenatal-preweaning) affect neonate, adolescent, and adult offspring behavior.

1.5. Preclinical overview of offspring exposed to prenatal morphine (& through lactation)

The following sections discuss behavioral effects observed in offspring in preclinical models of prenatal morphine exposure and describe some of the potential underlying cellular and molecular mechanisms. I also pay special attention to the duration of morphine exposure, as a possible explanation for discrepancies among studies. I focus on gestational-only exposure (pre-conception/GD 0 – end of

gestation/PND 0), partial gestation exposure, and gestation + lactation (maternal/pup) morphine exposure (Appendix Tables 1-3). Most studies reviewed use a partial morphine gestation paradigm (Appendix Table 2), begging further analysis on whether offspring outcomes are partially due to acute offspring withdrawal. The outcomes highlighted in the following sections relate to physical consequences of NOWS, changes in learning and memory-associated function, changes in affective behavior and stress hormones, and effects on drug-related reward.

1.5.1. Effects of morphine on physical characteristics & biochemical systems

Preclinical studies report no difference in litter size, offspring sex ratio, or mortality in pups born to dams treated with morphine during gestation (Chiang et al., 2014; Ramsey et al., 1993; Shen et al., 2016; Timár et al., 2010). Studies reporting no weight changes in offspring administered morphine to the dam either as an injection or orally, via forced morphine drinking (Appendix Table 1-3) (Ali Ahmadalipour et al., 2018; Che et al., 2005; Chiang et al., 2014; Dutriez-Casteloot et al., 1999; Gagin et al., 1997; Klausz et al., 2011; Shen et al., 2016; Tan et al., 2015; Torkaman-Boutorabi et al., 2019; Y. Wang, Yao, Nie, et al., 2017; Y. Wang, Yao, Li, et al., 2017). Studies directly comparing differences in offspring effects as they relate to differences in the timing, duration and route of maternal morphine administration have not been conducted systematically. One study by Timar *et al*. (2010) investigated the effects of varied length of maternal treatment, injecting female rats with morphine either 1) during gestation (prenatal) only, 2) during lactation (postnatal) only, or 3) throughout gestation and lactation. Compared to saline-exposed offspring, both male and female offspring from all

three morphine-exposed groups had decreased bodyweight at postnatal day 1 (PND 1), while their body weight at PND 14 and PND 21 exceeded the body weight of saline-exposed offspring. Other studies found that maternal morphine exposure throughout gestation and lactation resulted in lower offspring body weight (Eriksson & Rönnbäck, 1989; Siddiqui et al., 1997; Sobor et al., 2010).

There are several possible explanations for the discrepancies seen in preclinical studies examining the effect of prenatal morphine exposure on bodyweight. One possible explanation is that the most utilized preclinical models do not lead to high enough plasma concentrations of morphine to affect the fetus/newborn. Alternatively, because lower body weight is frequently observed in clinical studies but not in preclinical studies, it suggests that the stress associated with intermittent parental drug withdrawal might increase the vulnerability of the offspring. Finally, an extended maternal morphine treatment that continues throughout the lactation period might be necessary to recapitulate the clinical outcomes of prenatal morphine and NOWS when comparing various morphological and functional milestones relating to eye, cardiac, immune, and brain development (Clancy et al., 2001; Craig et al., 2003; Holsapple et al., 2003; Krishnan et al., 2014; Kroon et al., 2019; Lazic, 2012; Rice & Barone, 2000; Richard & Flamant, 2018; Van Cruchten et al., 2017). Due to the short gestation of rodents compared to humans, many processes like myelination and immune function continue developing postnatally in rodents (Craig et al., 2003; Holsapple et al., 2003). Therefore, opioid exposure during early rodent postnatal days might be an important consideration when developing maternal drug exposure paradigms.

Fetal brains begin to express opioid receptors around the end of the first trimester in humans (Magnan & Tiberi, 1989; Ray & Wadhwa, 1999) and the 14th

gestational day in rodents (Rius et al., 1991; Zhu et al., 1998). The effects of prenatal morphine on the endogenous opioid system include, among others, decreases in proenkephalin-derived opioid peptides (Schindler et al., 2004) and increases in μ- and δ-opioid receptor binding (Rimanoczy & Vathy, 1995; Ilona Vathy et al., 2003). Effects on μ-opioid receptor density appear to depend on multiple factors including the sex of the newborn, the brain region, and/or the developmental period examined. For example, PND 14 offspring who were exposed to morphine throughout gestation and lactation (GD 0- PND 30) displayed decreased μ-opioid receptor density in the striatum, thalamus, and amygdala, but not in the midbrain, NAc, hippocampus, or cortex (Chiou et al., 2003). Another study examining offspring exposed to morphine only during late gestation (GD 11-18) reported decreased μ-opioid receptor density in the basolateral and lateral amygdalar nuclei, an effect which persisted into adulthood (I. Vathy, 1995). However, it would appear that some of morphine's effects on development are sex specific as prenatally-exposed adult male offspring from the same study, but not females, showed increased μ-opioid receptor density in the NAc, the posteromedial cortical amygdala (PMCoA) and the central amygdala (CeA) when compared to control male offspring (Ilona Vathy et al., 2003). Overall, the literature suggests that prenatal morphine exposure produces long-term changes in μ-opioid receptor density that affect several brain regions, with potential repercussions on natural and drug reward mechanisms.

Similar to the opioid receptor system, the effects of *in utero* morphine exposure on other receptor systems depends both on the timing and duration of exposure, and the brain region considered. *In utero* exposure between GD 3-20 did not lead to changes in mRNA expression levels for dopamine receptors D1a, D2 and D3 in the NAc (Chiang et al., 2014). Although qualitative changes in receptor expression levels have been

documented, fewer studies describe whether those alterations have functional consequences. Morphine exposure during mid-late gestation (GD 7-21) led to a profound increase in adenylate cyclase activity stimulated by postsynaptic D1 dopamine receptors in fetal striatal slices (De Vries et al., 1991). The same treatment also increased noradrenaline release, suggesting an enhancement of the activity of dopaminergic and noradrenergic signaling *in utero* (De Vries et al., 1991). However, offspring born to females injected with low doses of morphine from GD 3-20 showed basal cAMP levels in the NAc that remained similar to control offspring, indicating that the impact of gestational morphine on the dopaminergic system may be transitory (Chiang et al., 2014). Effects on other neurotransmitter systems appear to be longer lasting. For example, rat offspring exposed to maternal morphine throughout gestation and lactation (GD 0- PND 30) exhibited deficits in AMPA receptor and NMDA receptor subunits, as well as post-synaptic density protein PSD-95 in NAc, VTA, and PFC at PND 14 and PND 30 (P. L. Wu et al., 2018), suggesting that long-term changes in neuroplasticity may have occurred as a result of morphine exposure *in utero*. Other effects related to neuroplasticity, including attenuation of long-term potentiation (LTP), long-term depression (LTD) and levels of proteins associated with synaptic transmission have also been noted in offspring after perinatal morphine exposure (Villarreal et al., 2008; Yang et al., 2003b)

Overall, it is clear that exposure to morphine *in utero* affects multiple neurotransmitter systems. More systematic studies are needed to determine both how persistent those changes are and the extent of their functional significance. Timing and length of dosing, route of administration, and dose of maternal morphine will undoubtedly impact molecular and behavioral outcomes and are likely to be the source

of some of the discrepancies observed in the literature. Moreover, because effects are often observed in several brain regions after *in utero* morphine exposure, it can be difficult to determine with confidence how a particular molecular effect contributes to a behavioral effect. Developing a greater understanding of the interplay between specific molecular, physical and behavioral changes will be key for identifying vulnerable developmental windows and for improving treatment of NOWS.

1.5.2. Effects on cognitive function

An increasing number of studies has demonstrated that children with prenatal opioid exposure might have increased risk for cognitive deficits (Table 1-1). Mild deficiencies in attention, complex working memory, and episodic memory have been reported when opioids are used chronically for pain relief (Kamboj et al., 2005; Sjøgren et al., 2005). Endogenous opioid peptides and their respective receptors are expressed in all hippocampal regions, including the dentate gyrus (DG), which is important for cognitive processes such as affect, mood, emotion, and learning and memory (Arvidsson et al., 1995; Kibaly et al., 2019; Taki et al., 2000). In the CA1 area of the hippocampus, opioid receptors show dense expression and the activation of μ-opioid receptors on interneurons in this region reduces their firing rate and subsequent neurotransmitter release rate, which in turn disinhibits pyramidal neurons, leading to increased excitation (Dacher & Nugent, 2011; Simmons & Chavkin, 1996). Interactions between the hippocampus and PFC are also important for episodic memory (Eichenbaum, 2017). The PFC expresses appreciable levels of μ-opioid receptors, and μ-opioid receptor signaling modulates PFC neuronal activity (Mitchell et al., 2012; Qu et al., 2015; Rola et al., 2008; Witkowski & Szulczyk, 2006). Investigators attempting to

determine whether pre- and/or perinatal morphine exposure results in cognitive impairments later in life have employed tasks that assess learning and memory.

1.5.2.1. Behavioral tests for cognitive function

Given that some clinical literature reports deficits in learning and memory in children exposed to pre- and perinatal morphine exposure, it is important to consider whether opioid exposure also increases vulnerability to learning and memory deficits in adult offspring. Nasiraei-Moghadam *et al.* (2013) found that only male offspring who had been exposed to morphine throughout the entire gestation period (GD 1-21) displayed memory deficits during adolescence. Interestingly, offspring exposed to morphine during early-mid gestation (GD 1-13) showed no memory deficits (Akbarabadi et al., 2018), further supporting the notion that the length and timing of maternal drug dose is critical for determining offspring behavioral outcomes. In addition, female offspring displayed memory deficits that started during adolescence and persisted into adulthood, an effect that was observed regardless of the duration of gestational morphine exposure (Nasiraei-Moghadam et al., 2013). Together, these studies suggest that females may be more susceptible to memory impairments than males and point to the need for more studies examining sex-specific differences in learning and memory following pre- and perinatal morphine exposure.

There are several behavioral tests that can be used to study unique aspects of learning and memory, including the Morris Water Maze (MWM) (D'Hooge & De Deyn, 2001). While several studies found no deficits in spatial learning during the final test phase in the MWM, morphine-exposed offspring tended to show greater impairment in training and acquisition phases of learning (Ali Ahmadalipour et al., 2018; Šlamberová et

al., 2001; Yang et al., 2003b). Interestingly, these effects could be reversed with exercise or environmental enrichment, suggesting the importance of environmental stress in perpetuating cognitive deficits (Ali Ahmadalipour et al., 2018). Sex differences have also been reported in some hippocampal-dependent behavioral tests. When a symmetrical maze was used to measure learning, adult females exposed to morphine during mid-gestation (GD 11-18) displayed no deficits, while male morphine-exposed offspring actually completed the task faster than controls, suggesting a possible hyperlocomotive effect (Šlamberová et al., 2001). When the same group of animals was tested in the eight-arm radial maze, which assesses working spatial memory, both male and female morphine-exposed offspring required more time to complete trials than controls (Šlamberová et al., 2001).

Overall, the literature suggests that prenatal morphine exposure can produce persistent learning and memory deficits that might confer vulnerability to memory-related neuropsychiatric disorders in adulthood.

1.5.2.2. Potential mechanisms for cognitive deficits

In utero morphine is capable of altering the function of neurotransmitter systems, including the endogenous opioid system (Rimanoczy & Vathy, 1995; Ross et al., 2015). One study found increased μ-opioid receptor binding in hippocampal DG slices from offspring exposed to maternal morphine throughout gestation and lactation (Šlamberová, Bar, et al., 2003). A separate study examined protein and mRNA levels of opioid precursors in the DG of offspring exposed to morphine during mid-gestation (GD 11-18) and found decreased proenkephalin/met-enkephalin levels, but increased

prodynorphin/dynorphin levels (Schindler et al., 2004). This is in line with previous studies which have shown that opioid withdrawal in adult rodents downregulates proenkephalin and upregulates prodynorphin (Bali et al., 2015) and further supports the notion that endogenous opioids are critical for regulating neuronal processes within the hippocampus (Harburg et al., 2007; Kibaly et al., 2019).

Alterations to synaptic transmission and neuroplasticity are also a likely source of the cognitive deficits observed following *in utero* morphine exposure. Notably, in hippocampal slices from adult male offspring exposed to morphine during mid-gestation (GD 11-18) there was a lower baseline excitatory postsynaptic potential (EPSP) slope compared to saline offspring (Velíšek et al., 2003). In addition, there was no change in EPSP slope after high-frequency stimulation (HFS), suggesting that both basal synaptic transmission and plasticity were impaired (Velíšek et al., 2003). Other studies in hippocampal slices from mice exposed to pre- and/or perinatal morphine have found similar results including impaired LTP induction and population spike amplitude (Ali Ahmadalipour et al., 2018; Sarkaki et al., 2008), and impaired LTD after low-frequency stimulation (Yang et al., 2003a). Even in chicks exposed to morphine from embryonic day 5-8, there are deficits in LTP and paired-pulse facilitation in a brain region that is comparable to the hippocampus (J. Jiang et al., 2011). Interestingly, though contextual fear-conditioning impaired HFS-induced LTP in control offspring, both LTP and LTD were resistant to change in offspring exposed to morphine during mid-gestation (GD 9-18), suggesting that normal stress responses were blunted (Tan et al., 2015).

Morphine can also alter expression of endogenous neurotrophins, like brain-derived neurotrophic factor (BDNF), which has been implicated in memory consolidation and addiction (Barker et al., 2015; Li et al., 2017). Decreased BDNF was observed in the

hippocampus of adolescent and adult female offspring exposed to morphine for varied lengths of time (GD 1-13, GD 11-18, GD 1-21), though the phenomenon could be reversed with exercise or postnatal enrichment (Ali Ahmadalipour et al., 2018). This effect appeared to be sex-specific, given that no adolescent or adult male offspring, from any of the morphine-exposed treatment groups, showed changes in precursor BDNF protein levels in the hippocampus. These studies further highlight the multiplexed way in which sex and the duration and timing of drug exposure interact to produce unique effects on neuronal development and plasticity.

1.5.3. Effects on affective behaviors and stress hormones

Studies have demonstrated that prenatal stressors, including drug exposure, can alter the functionality of the Hypothalamic-Pituitary-Adrenal (HPA) axis, the system primarily responsible for mediating the natural stress response (Bali et al., 2015; Burke & Miczek, 2014; T. A. Kosten & Ambrosio, 2002). Stress has been shown to cause HPA axis dysfunction, leading to changes in cortisol levels in humans and corticosterone levels in rodents, which is dependent on many factors, including -but not limited to- the type of stressor, the time of day, and variability in susceptibility and resiliency to the stressor (Belda et al., 2020; Ceruso et al., 2020; Gururajan et al., 2019; Jacinto et al., 2017; Lowrance et al., 2016; Ostrander et al., 2006; Piskunov et al., n.d.; Tripathi et al., 2017; Yohn et al., 2019). Morphine itself has also been reported to impact corticosterone levels, though this effect is not well understood and likely depends on the diurnal cycle and on hormone levels as well (Kiem et al., 1995; Koebele & Bimonte-Nelson, 2016). A growing body of preclinical literature has examined the effect of *in utero* morphine

exposure on behaviors related to stress and relevant to psychiatric diseases including anxiety-like behavior, compulsive-like behavior, and depressive-like behavior.

1.5.3.1. Tests for stress-related behaviors

To our knowledge, there is currently no clinical literature that has reported changes in anxiety and/or anxiety-associated stress responses in children that were treated for NOWS during infancy. However, preclinical studies have begun to shed light on the long-term behavioral consequences of maternal morphine exposure. Interestingly, adult offspring who were exposed to morphine *in utero* (GD 1-21) displayed decreased anxiety-like behavior in both the Elevated Plus Maze (EPM) and the Light-Dark Box (LDB), possibly indicating a hypo-functionality of the HPA axis (Tan et al., 2015). However, a separate study, which exposed offspring to morphine throughout gestation and lactation (GD 1- PND 21) found no differences in anxiety-like behavior in the EPM (Klausz et al., 2011). Like other behavioral effects observed following morphine exposure, it appears that several variables, including timing and duration of exposure, and sex, contribute to alterations in anxiety-related behaviors.

To further investigate anxiety-like behavior, preclinical studies also utilize tests that measure locomotion and the response to novel environments. Regardless of the timing and duration of *in utero* morphine treatment (gestation only, lactation only, gestation and lactation), Timar *et al.* found that peri-adolescent (PND 23) male offspring displayed increased locomotor activity in the Open Field Arena (OFA) compared to controls, suggesting slower habituation to a novel environment (Timár et al., 2010). When male mice were exposed to morphine during mid-gestation (GD 11-18) followed

by postnatal exposure to a cold stressor for 14 days, they displayed decreased locomotion and increased thigmotaxis in the OFA compared to non-treated, non-exposed offspring (Šlamberová et al., 2002). However, when exposed to *in utero* morphine for a similar duration (GD 9-18) but in the absence of a postnatal thermostressor, male adult offspring displayed no differences in locomotion compared to controls (Tan et al., 2015). It is especially important to consider sex-specific differences in stress and anxiety given that estrogen is known to modulate the HPA axis (Koebele & Bimonte-Nelson, 2016). Regardless of timing and duration of prenatal morphine treatment, ovarectomized females did not show differences in the OFA while morphine-exposed females treated with an estrogen precursor, estradiol-3-benzoate (EB), showed increased anxiety-like behavior when compared to EB-treated controls (Šlamberová et al., 2002). These data highlight the many factors that complicate the study of stress and anxiety, including interactions between multiple types of stressors and the prominent role of sex hormones in regulating natural stress responses.

Similarly, there is only one clinical study to our knowledge that has reported on the prevalence of mood disorders, like major depressive disorder (MDD), in children who were exposed to gestational morphine (Nygaard et al., 2020). However, many preclinical studies have examined depressive-like behavior, behavioral despair, and anhedonia in rodents, which are considered signature correlates of human behaviors commonly seen in clinical depression (Table 1-1). For example, when exposed to morphine throughout gestation and lactation (GD 1- PND 21), both adolescent and adult offspring show increases in depressive-like behavior in the Forced Swim Test (FST) (Klausz et al., 2011). Like anxiety-related behaviors, a sex-specific effect can also be observed when studying depressive-like behavior. Notably, ovariectomized females, regardless of duration and timing of prenatal morphine treatment, showed no differences in the FST

while morphine-exposed females treated with EB showed increased depressive-like behavior, measured by more time spent floating, in the FST (Šlamberová et al., 2002). Taken together, these studies indicate that pre- and/or perinatal morphine exposure may alter stress-reactivity, thereby contributing to pathological behaviors implicated in psychiatric diseases like anxiety and depression.

1.5.3.2. Hormones affecting stress reactivity and HPA axis functionality

Decreases in neonatal brain volume have been reported following stressors, including prenatal drug exposure, as have changes in organ weight, which may have implications for stress-induced physiological and behavioral responses (Gui et al., 2019; Sirnes et al., 2017; Upadhyay et al., 2010). Adolescent and adult offspring exposed to morphine throughout gestation and lactation (GD 1- PND 21) displayed a decrease in thymus weight, spleen weight and adrenal weight (Klausz et al., 2011). The effects of late gestation (GD 11-18) morphine exposure on adrenal weight are less clear with one study reporting a decrease in weight but others reporting no changes in weight (Dutriez-Casteloot et al., 1999; Laborie et al., 2005; J. Lesage et al., 1996). In the study which reported decreased adrenal weight after late-gestation exposure, the effect disappeared when mothers were also adrenalectomized, perhaps highlighting the importance of maternal stress hormones in mediating the previously observed deficits (Jean Lesage et al., 2000).

An activated stress response also entails the release of corticotropin-releasing factor (CRF) from the hypothalamus, which signals to the pituitary to release adrenocorticotropic hormone (ACTH), and ultimately promotes the release of cortisol

from the adrenal cortex (Goldstein, 2010). Cortisol remains elevated for several hours following a stressor but eventually acts as a negative-feedback modulator within the hypothalamus, inhibiting further CRF release (Hannibal & Bishop, 2014). With repeated, chronic stress, cortisol homeostasis becomes dysregulated and stress hormone levels remain persistently high (Qin et al., 2016). In particular, levels of CRF affect the response to stressors and their dysregulation has been implicated in depression, psychiatric diseases, and negative affect during drug withdrawal (Y. Jiang et al., 2019). Offspring exposed to morphine during mid-late gestation (GD 11-18) however did not display changes in hypothalamic CRF levels (Dutriez-Casteloot et al., 1999). This indicates that observed increases in anxiety-like behavior following *in utero* drug exposure are not due to changes in CRF, which suggests that further studies must be conducted to probe if these offspring have a dysregulated stress system affecting various brain regions and possibly downstream hormonal production.

Changes to the stress system following *in utero* morphine exposure have also been observed at the level of ACTH, which is the hormone released from the pituitary gland in response to stimulation by hypothalamic CRF. When exposed to morphine throughout gestation and lactation (GD 1- PND 21), both adolescent and adult offspring had lower levels of ACTH (Klausz et al., 2011), but when exposed only during mid-late gestation (GD 11-18) their ACTH levels were normal (Dutriez-Casteloot et al., 1999; Rimanóczy et al.,2003 ; Laborie et al., 2005). Interestingly, when exposed to morphine during both gestation and lactation (GD 1- PND 21) and then exposed to a mild (EPM) and a severe stressor (FST), adult and adolescent offspring showed lower levels of ACTH compared to saline-control (Klausz et al., 2011). Decreased levels of ACTH were also observed after a 20-minute restraint stress in adult male offspring exposed to morphine only during mid-late gestation (GD 11-18) (Rimanóczy et al., 2003), though

decreases were not apparent after only a 3-minute ether-inhalation stress, suggesting that the type and duration of stress impacts HPA axis response (Laborie et al., 2005). Female offspring exposed to morphine during gestation also showed lower levels of ACTH compared to controls regardless of whether they were in the diestrus (low estrogen levels) or proestrus (high estrogen level) phase of the cycle (Šlamberová et al., 2004). This further highlights how *in utero* morphine exposure could potentially lead to hypo-activity of the HPA axis.

Following ACTH release, cortisol is synthesized and released from the adrenal cortex where it circulates throughout the body and brain, stimulating a variety of effects. Because the primary HPA axis hormones- CRF, ACTH, cortisol- act in a negative feedback loop, it follows that alterations to one component will result in imbalance of the entire system. As such, dysregulation at the level of cortisol has also been observed following pre- and perinatal exposure to drugs like morphine. Under basal conditions, adolescent offspring exposed perinatally (GD 1- PND 21) to morphine, displayed lower corticosterone (the rodent correlate of cortisol) levels, but as they aged into adulthood, levels became higher than controls (Klausz et al., 2011). In contrast, there was no difference in basal corticosterone levels in offspring exposed to morphine only during the end of gestation (GD 11-18) (Dutriez-Casteloot et al., 1999; Laborie et al., 2005; Rimanóczy et al., 2003). After a mild stressor (EPM) or 3-minute ether-inhalation, corticosterone levels in adolescent and adult rats exposed to perinatal (GD 1 – PND 21) or mid-late gestational (GD 11-18) morphine, were unchanged, but, after a severe stressor (FST) levels in perinatal-exposed offspring were decreased compared to controls, further supporting the notion that the type and duration of stressor is critical in determining HPA axis hormonal response (Laborie et al., 2005; Klausz et al., 2011). When exposed to morphine during mid-late gestation (GD 11-18), neither adult male,

diestrus female, or proestrus female offspring showed changes in corticosterone levels after a 20-minute restraint stress, indicating that length and timing of morphine exposure also plays a role in HPA axis regulation (Rimanóczy et al., 2003; Šlamberová et al., 2004).

The HPA axis is also influenced by various neurotransmitter systems located throughout multiple brain regions, and imbalance of these systems can lead to dysregulation of the HPA axis (Burke & Miczek, 2014; Mokrani et al., 1997; Ornoy & Koren, 2019; Rodgers et al., 2013). It has been reported that *in utero* opioid exposure leads to changes not only in the noradrenergic system but also in the dopaminergic and serotonergic systems, as well as the aforementioned endogenous opioid system (Byrnes & Vassoler, 2018; Hendrée E. Jones & Kraft, 2019; Weller et al., 2020). In hippocampal tissue from offspring exposed to morphine during mid-late gestation (GD 11-18), levels of serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were not different at baseline but increased substantially more than control levels after ether-inhalation stress (Laborie et al., 2005). In the hypothalamus of these offspring, 5-HT and 5-HIAA content were higher at baseline and showed longer latency to decrease after ether-inhalation stress when compared to controls (Laborie et al., 2005). In the future, it will be critical to conduct studies which directly examine the relationship between changes to neurotransmitter systems and changes to the HPA axis, in order to allow for a more mechanistic evaluation of the effect of pre- or perinatal morphine exposure on stress-reactivity.

Overall, both behavioral and molecular data suggest that *in utero* morphine can alter offspring stress response, which may contribute to the development of psychiatric disorders later in life.

1.5.4. Effects on drug-related reward and lifetime intake of opioids and non-opioid drugs

Opioids, like other recreational drugs, act on the mesocorticolimbic system to exert their rewarding effects (Ford et al., 2006). With continuous drug use and through multiple iterations of intoxication and withdrawal, maladaptation occur at multiple levels of this circuitry, which perpetuate the cycle of misuse (Adinoff, 2004; Koob & Volkow, 2016). However, it remains unclear exactly how prenatal and perinatal opioid use changes the functionality of the developing mesocorticolimbic system, how persistent these changes may be, and whether they contribute to observable behavioral outcomes. A common hypothesis is that prenatal morphine exposure produces neurochemical alterations which lead to increased vulnerability of the offspring to develop drug addictions later in life. As previously mentioned, in humans, susceptibility to developing a drug use disorder is heavily influenced by stressors in the environment, socioeconomic status, and co-occurring psychiatric disorders, among others. Although those influencing factors cannot be easily modelled, preclinical studies do suggest that maternal morphine use affects subsequent drug use by adult offspring later in life. These studies utilize common forms of measuring reward sensitivity in preclinical models, including conditioned place preference (CPP), drug self-administration, behavioral sensitization measured by locomotion, and oral drug consumption measurements (Stephens et al., 2010).

1.5.4.1. Effects on opioid-related reward

A number of preclinical studies have examined how pre- or perinatal exposure to morphine affects opioid drug use in adolescent and adult offspring. For example, one study in rats found that offspring exposed to morphine during mid-gestation (GD 12-18)

displayed increased preference score in morphine-induced CPP compared to controls (Gagin et al., 1997). Similarly, studies in chicks found that morphine injected during late embryonic days also produced morphine-induced CPP (He et al., 2010; Y. Wang, Yao, Li, et al., 2017) and increased locomotion (Y. Wang, Yao, Li, et al., 2017). In addition, after pre- and perinatal morphine exposure (GD 1- PND 21), both male and female offspring displayed morphine-induced CPP at sub-threshold doses (1 mg/kg or 3 mg/kg) (Timár et al., 2010). Gestation-only (GD 1-21) exposure also resulted in morphine-induced CPP and morphine-induced behavioral sensitization in male offspring, as well as increased dopamine and serotonin turnover rates in the NAc after a sub-threshold dose of morphine (L. Y. Wu et al., 2009). Together, this suggests both a greater positive reinforcing effect of morphine and an increased sensitivity of the mesolimbic pathway in offspring after pre- and/or perinatal morphine exposure.

Other studies, however, have reported no significant offspring differences in morphine CPP following parental morphine exposure. With a lower dose of morphine, offspring exposed to morphine prenatally did not show CPP (Akbarabadi et al., 2018; J. Jiang et al., 2011; Riley & Vathy, 2006; Sadat-Shirazi et al., 2019). It is possible that prenatal or parental morphine exposure triggered adaptations in these offspring that resulted in tolerance to the reinforcing properties of morphine. For example, increased monoamine oxidase (MAO) in the NAc was noted in one group of prenatally exposed offspring, which may have increased dopamine degradation and thereby decreased reward in response to the drug (Sadat-Shirazi et al., 2019). However, when morphine reward was tested in either a two-bottle (Vousooghi et al., 2018) or three-bottle choice (Sadat-Shirazi et al., 2019), prenatally-exposed offspring did show increased preference, suggesting that the paradigm used to test drug reward is a key factor in determining if differences across treatment groups will be observed.

Mixed results have also been reported following intravenous (IV) opioid self-administration (SA) during adulthood. Adult male offspring exposed to morphine partially during gestation (GD 7- birth) self-administered for heroin a greater number of times than control offspring (Ramsey et al., 1993). Similarly, female offspring who were exposed to morphine throughout gestation and lactation (GD 7- PND 21) self-administered for morphine more than controls, but only during the first week of the paradigm (Glick et al., 1977). Interestingly, when exposed to morphine only during mid-late gestation (GD 11-18), adult male offspring did not show differences in total morphine intake at 0.3, 2, or 3 mg/kg/infusion dose, but did show increased active lever presses at the 1 mg/kg dose (Ramsey et al., 1993; Riley & Vathy, 2006). Together, this suggests that offspring outcomes depend on the *in utero* morphine paradigm used and the behavioral procedure used to study offspring's response to opioids misuse in adulthood.

1.5.4.2. Effects on methamphetamine- and cocaine-related reward

Studies have shown that pre- and perinatal morphine exposure also affects non-opioid drug misuse, primarily stimulants like methamphetamine and cocaine, later in life. For example, after *in utero* (GD 3-20) exposure, male adult offspring showed methamphetamine (METH)-induced CPP at 2 mg/kg (Shen et al., 2016) and 0.5 mg/kg (Chiang et al., 2014) at similar levels to control offspring, but at higher doses required more time and higher number of sessions to extinguish METH-CPP. Offspring also displayed METH-priming-induced reinstatement in CPP, suggesting an increased vulnerability to drug relapse (Shen et al., 2016). When tested in a behavioral sensitization paradigm, male offspring exposed to morphine partially during gestation

(GD 3-20) did not show differences in locomotion compared to controls, showing that METH-induced sensitization was unaffected (Chiang et al., 2014; Kuhn et al., 2019). In a METH-IV self-administration model, male adult offspring who were exposed to partial-gestational morphine (GD 3-20) showed no difference in fixed ratio (FR) or progressive ratio (PR) lever presses and no differences in break point. However, as with the METH-CPP described above, partial gestational morphine exposure did result in slowed extinction and increased lever pressing during METH-priming induced, but not cue-induced, reinstatement. Moreover, these changes were specific to methamphetamine as no differences were reported in the acquisition, extinction, and reinstatement of food self-administration (Shen et al., 2016).

Although the interaction of morphine and cocaine has been studied (Velazquez et al., 2010), few have investigated how prenatal morphine affects risk for cocaine use in adulthood. One study found that adult male offspring who were exposed to morphine during mid-late gestation (GD 7- birth) self-administered for cocaine a greater number of times than controls, with no change in the number of saline infusions, suggesting the effect was drug-specific and not the result of hyperlocomotion (Ramsey et al., 1993). However, male and female offspring that were exposed to morphine during mid-late gestation (GD 11-18) showed no difference in the number of active lever presses for cocaine in an IV FR1 self-administration schedule (I. Vathy et al., 2007). Like studies examining opioid-misuse risk in adulthood following prenatal morphine exposure, these studies highlight the way in which differences in maternal opioid exposure and offspring drug reward paradigm, among other factors, may explain some of the discrepancies seen in behavioral results, especially as they relate to vulnerability to develop a drug use disorder.

It is still unknown how prenatal and perinatal morphine exposure affects offspring's misuse of drugs that are more commonly encountered during adolescence and adulthood, including nicotine, cannabis, and ethanol. The experiments designed in Chapters 2-3 investigate how prenatal-postnatal and prenatal-preweaning morphine exposure affects adult ethanol non-operant self-administration. Furthermore, Chapter 4 investigates how prenatal-preweaning morphine exposure affects adolescent nicotine use, and how that adolescent drug exposure alters subsequent adult ethanol binge-like drinking.

1.6. Overall considerations

Using varied maternal drug administration paradigms, preclinical studies have been able to recapitulate some aspects of the physical symptoms of NOWS seen in human neonates. Preclinical studies have also found deficits in behavior, like learning and memory, obsessive-like behavior, anxiety-like behavior, and depressive-like behavior, which persist into adulthood and may affect development of psychiatric disorders. A growing number of preclinical studies have also attempted to elucidate brain region-specific molecular mechanisms that could cause disruption of synaptic plasticity, stress reactivity, and reward.

Overall, prenatal opioid exposure affects offspring behavior and neurodevelopment, seen acutely with NOWS, but also extending throughout adolescent development and even later in life. I reviewed the preclinical literature for studies investigating outcomes focusing on physical, molecular, and behavioral outcomes for offspring after gestational or perinatal morphine exposure. The variability in findings is likely attributed to both the

inherent vulnerability and/or resiliency in sub-populations of offspring, but also to the many differences in maternal drug exposure paradigms. These differences include dose of drug, duration and timing of exposure, and route of drug administration. This not only imparts pharmacokinetic and pharmacodynamic inconsistencies, but also differentially impacts the stress experienced by the dam during treatment. Similar discrepancies tend to pervade retrospective clinical studies and can make cross-study comparisons difficult, if not impossible. For these reasons, future systematic and standardized methodology in maternal and neonatal opioid administration is needed.

1.7. Thesis objectives

The goal of this dissertation was to investigate the effects of maternal morphine exposure on neonate, adolescent, and adult offspring behavior. I validated an oral, voluntary morphine paradigm for dams to investigate offspring outcomes after two critical periods: prenatal-perinatal and prenatal-preweaning morphine exposure. The studies presented here show evidence for age- and sex-specific alterations in offspring after each exposure period, related to affective behavior and drug intake misuse liability in vulnerable sub-populations of offspring. This dissertation's aim was to investigate the following three topics:

1. Prenatal-perinatal opioid exposure and its behavioral consequences in cross-fostered mice offspring
2. Effects of prenatal-preweaning morphine exposure on behavior in mice offspring

3. Effects of prenatal-preweaning morphine exposure on adolescent nicotine use risk and consequently adult ethanol binge drinking

Table 1-1. Pathophysiology and consequences of human NOWS with translational behavioral correlates in preclinical rodent models that can be used to identify rodent offspring vulnerability.

	Human NOWS	**Experimental rodent behavioral correlates**
Acute Physical NOWS Manifestations Adapted from: (Maguire et al., 2016; Weller et al., 2020)	Disturbed sleep patterns	EEG (Rensing et al., 2018) EMG (Rensing et al., 2018) Home-cage activity recording using high definition camera
	↓ body weight (Bakhireva et al., 2019)	Weigh offspring throughout development
	↓ head circumference	Measure head circumference using calipers
	Excessive sucking	Suckling test (Calamandrei et al., 1991)
	Loose stools/Vomiting	N/A
	Tremors/Seizures	EEG (Chemaly et al., 2018; Sampath et al., 2014)
	Skin injuries (ex. diaper dermatitis, scratches)	Skin injuries can be detected in adolescent/adult mice (Burkholder et al., 2012)
	Irritability	N/A
	High-pitched crying	USVs

		(emotional development) (Baharnoori et al., 2012; Hahn & Lavooy, 2005)
	↑ muscle tone	nuchal EMG (Blumberg et al., 2015; Seelke & Blumberg, 2005)
	Hyperthermia	Subcutaneous temperature probes (Grimaud & Murthy, 2018; Seelke & Blumberg, 2005)
	↑ respiratory rate	Plethysmography (Gulemetova & Kinkead, 2011) EMG recordings of respiratory muscle (Grimaud & Murthy, 2018)
	Nasal congestion	N/A
Auditory & Visual Manifestations Adapted from: (Maguire et al., 2016; Weller et al., 2020)	Otitis media & auditory deficits post-otitis media	Auditory startle response in pups (Baharnoori et al., 2012; Fox, 1965) Pre-pulse inhibition response Otoscopic examination (MacArthur & Trune, 2006)
	↓ visual acuity	ERG & (Benchorin et al., 2017; J. Zhang et al., 2005) Spatial learning tasks that requires intact visual system – will be strain dependent due to cone degeneration in adult mice (Morris Water Maze, Conditioned Place Preference, Contextual Fear Conditioning)

	Strabismus	N/A
	Nystagmus	N/A
Sensory-Motor Impairments Adapted from: (Maguire et al., 2016; Weller et al., 2020)	↓ score in Bayley Mental Developmental Index & ↓ score in Psychomotor Developmental Index	Nest-seeking Behavior (olfactory discrimination in pups) (Baharnoori et al., 2012) Odor-stroke Associative learning task (in pups) (Baharnoori et al., 2012) EOG (Z. Wang & Storm, 2011)
	↓ self-regulation (Bakhireva et al., 2019)	N/A
	↓ 'typical performance' on sensation-seeking scale in ITSP (Bakhireva et al., 2019)	N/A
	↓ motor skills	*Developmental/Reflex Milestones in pups:* Righting reflex Forelimb grasp reflex Cliff avoidance Negative geotactic reaction Grip strength response (Baharnoori et al., 2012; Fox, 1965; Nguyen et al., 2017; Rice & Barone, 2000; Sampath et al., 2014)
	Hyperactivity	*Locomotion tests:* Open Field Arena (novel environment) (Baharnoori et al., 2012)

		Home cage activity using infrared beams Rotarod Horizontal ladder crossing test
	Attention deficits	Combination of genetic/pharmacological manipulations assessed using variety of behavioral tests, including 5-choice serial reaction time (Russell, 2011)
Learning & Memory Deficits Adapted from: (Maguire et al., 2016; Weller et al., 2020)	↓ score on Columbia Mental Maturity scale & 	*Spatial Memory Tests:* Morris Water Maze (Crawley, 1999; Vorhees & Williams, 2014) Y Maze Test (Vorhees & Williams, 2014) T Maze Test (Vorhees & Williams, 2014) Barnes Maze Test Star Maze (Vorhees & Williams, 2014) Hole-board Maze (Vorhees & Williams, 2014) Object Location Memory Test (Conrad, 2010) *Fear Learning/Memory Tasks:* Contextual & Cue Fear

		Conditioning Passive Avoidance Test (Campos et al., 2013)
	↓ score on Cognitive Index & ↓ score on Memory Index	*Reference & Working Memory Task:* Radial Arm Maze (Vorhees & Williams, 2014) *STM/LTM Task:* Novel Object Recognition Test (Conrad, 2010)
	↓ score on Perceptual Index	N/A
	↓ performance IQ scores	N/A
	↓ score on NAPLAN standardized test	N/A
Affective Behavior Adapted from: (Maguire et al., 2016; Weller et al., 2020)	↑ risk to be hospitalized for psychiatric disorders	*Anxiety-related Tests:* Elevated Plus Maze Light-Dark Box (Bailey & Crawley, 2009; Campos et al., 2013; Crawley, 1999) *Behavior helplessness-related Tests:* Forced Swim Test Tail Suspension Test (Crawley, 1999)

		Other Affective Tests: Grooming Splash Test Nest-Building Behavior Marble Bury Test Somatic Signs
	Impulsivity	*Impulsivity Tests:* Delay discounting task Effort discounting task Five-choice or three-choice serial reaction time (Dent & Isles, 2014; Sasamori et al., 2018) Stop-signal task Go/No-go task (Dent & Isles, 2014)
	Aggression	*Social Behavior Tests:* Social dominance test Social interaction/preference test (Crawley, 1999; Golden et al., 2019) Sexual behavior (Crawley, 1999) Maternal Behavior (Baharnoori et al., 2012)
Risk of Future Drug Misuse	No conclusive studies yet	*Drug Reinforcement Tests:* Conditioned Place Preference Drug Discrimination Tasks Locomotor Sensitization

		Intravenous Self-Administration Drug Intake/Preference in a Bottle Choice (Crawley, 1999; Panlilio & Goldberg, 2007) *Negative Affect Tests:* Conditioned Place Avoidance Somatic Signs during drug withdrawal

Table 1-1: Abbreviations:

EEG: electroencephalography, **EMG**: electromyographic**, EOG**: electro-olfactogram**,** ERG: electroretinography, **ITSP**: Infant/Toddler Sensory Profile**, NAPLAN**: National Assessment Program-Literacy and Numeracy**, STM**: Short-term memory**, LTM**: Long-term memory

CHAPTER 2: In Utero Exposure to Morphine Leads to Sex-specific Alterations that Persist into Adulthood in Cross-fostered Mice

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In utero exposure to morphine leads to sex-specific behavioral alterations that persist into adulthood in cross-fostered mice

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2.1. Introduction

Opioid Use Disorder (OUD) is a major public health concern. The 2019 National Survey on Drug Use and Health reported that 3.7% of individuals aged 12 and older, including women of childbearing age, have misused prescription and/or illicit opioids (Lipari & Park-Lee, 2020). In addition, a 2015-2016 study showed that one third of pregnant women used opioids (St. Marie et al., 2020). Continued opioid use in pregnant women can lead to serious maternal, fetal, and neonate complications and, in extreme cases, can lead to death (Leyenaar et al., 2021; Ostrea et al., 1997).

Some newborns prenatally exposed to opioids display a series of symptoms categorized as Neonatal Opioid Withdrawal Syndrome (NOWS). A study of maternal-infant dyads prenatally exposed to opioids reported that roughly 30-60% of newborns were diagnosed with NOWS (Leyenaar et al., 2021; Skumlien et al., 2020), which suggests that a proportion of newborns exposed to opioids *in utero* may develop a phenotype severe enough to require pharmacological and/or non-pharmacological interventions. Characteristic manifestations of NOWS include decreased body weight, high-pitched crying, irritability, tremors, and an inability to be soothed, among many others (Piccotti et al., 2019; Weller et al., 2020). Reviews and meta-analyses of clinical data have reported infant-adolescent outcomes associated with *in utero* opioid exposure, including lower scores in neurocognitive and developmental assessments, decreased motor skills, and increased hyperactivity and aggression (Maguire et al., 2016; Minnes et al., 2011; Nygaard et al., 2017; Weller et al., 2020; Yeoh et al., 2019). However, some studies report variability in outcomes among children exposed prenatally to opioids, leading to potential differences in vulnerability and resiliency in these individuals (Labella et al., 2021; Sarfi et al., 2021). In addition, the long-term effects of maternal opioid use

on human offspring are not fully understood, including the vulnerability to develop a psychiatric disease, and the risk of drug misuse.

Preclinical models have been used extensively to study the long-term effects of maternal morphine exposure. Several rodent studies have reported the effects of prenatal and perinatal morphine exposure on offspring outcomes, including body weight, mortality, and organ size (Ahmadalipour et al., 2018; Chiang et al., 2014; Eriksson & Rönnbäck, 1989; Glick et al., 1977; Klausz et al., 2011; Ramsey et al., 1993; Shen et al., 2016; Siddiqui et al., 1997; Sobor et al., 2010; Tan et al., 2015; Timár et al., 2010). However, very few of them have examined other aspects of NOWS, such as high-pitched crying. Similar to the studies in humans, preclinical models of *in utero* morphine exposure report discrepant offspring outcomes. Many factors could contribute to such variability, including differences in maternal opioid exposure paradigms, including length of exposure and dose used, and whether dams experienced varying levels of gestational stress, like being shipped while pregnant or receiving daily injections. To date, no published study has used an oral two-bottle choice (2BC) morphine self-administration protocol for the maternal dam exposure, which minimizes any confounds associated to stress.

The preclinical literature has also shown conflicting results on whether parental morphine causes alterations in behavior related to psychiatric disorders, including anxiety-like, compulsive-like, and depressive-like behavior. For example, adult rodents exposed to morphine *in utero* displayed decreased anxiety in both the Elevated Plus Maze (EPM) and the Light-Dark Box (Tan et al., 2015). In contrast, a study where morphine was given between gestation day (GD) 1 and postnatal day (PND) 21, found no differences in offspring's anxiety-like behavior in the EPM (Klausz et al., 2011).

Contradictory findings highlight how the length of the dam's morphine exposure can affect offspring's baseline behavioral manifestations. Additionally, most studies have only examined a few offspring behaviors at a time, and to date, no study has evaluated behaviors with a comprehensive approach, to better define baseline phenotypes of adolescent and adult offspring after maternal opioid exposure.

Parental substance use disorder (SUD) and prenatal drug exposure is associated with a myriad of negative outcomes in offspring, including vulnerability to develop a SUD (Dodge et al., 2019). Negative outcomes in the offspring depend on the timing of the parental exposure to drugs and/or other external factors, like stress (Betcher et al., 2019; Biederman et al., 2000; Dodge et al., 2019; Glantz & Chambers, 2006; Madras et al., 2019; Nakhjiri et al., 2017; Peleg-Oren & Teichman, 2006; Tarter et al., 2020). Pre- and perinatal opioid exposure can alter offspring's predisposition to future drug use, including risk for the same drug-class from maternal exposure (i.e. morphine) or cross-tolerance to other drugs (i.e. cocaine and methamphetamine) (Chiang et al., 2014; Gagin et al., 1997; Glick et al., 1977; Ramsey et al., 1993; Shen et al., 2016; Timár et al., 2010; Vousooghi et al., 2018; L. Y. Wu et al., 2009). To our knowledge, no preclinical study has examined potential changes in alcohol intake and preference in offspring exposed to *in utero* morphine, even though alcohol is the most commonly used drug according to the 2019 National Survey on Drug Use and Health (Lipari & Park-Lee, 2020). In addition, drugs of misuse, including alcohol, can disrupt the functionality of the Hypothalamic-Pituitary-Adrenal (HPA) axis, a system important for regulating stress, and this can contribute to alcohol misuse and dependence (Blaine et al., 2016; Stephens & Wand, 2012). One clinical longitudinal study reported that a significantly higher proportion of adults whose mothers used heroin during pregnancy misused alcohol during their

lifetime (Nygaard et al., 2020). Furthermore, there is well-documented evidence for opioid-ethanol interactions (Arias & Kranzler, 2008; Gianoulakis, 2001; Gianoulakis et al., 1989; Job et al., 2007), warranting further investigation into how pre- and perinatal opioid exposure affects alcohol use risk, and if stress exacerbates these effects.

In the present study, we utilized a translational maternal morphine paradigm, where dependent dams orally self-administered morphine in a 2BC paradigm throughout pregnancy and during the first postnatal week to model morphine exposure during early and mid-gestation in humans (Richard & Flamant, 2018; Semple et al., 2013). We examined offspring in a battery of behaviors throughout adolescence and adulthood to compile a comprehensive behavioral score. After adolescent testing, offspring were monitored for alcohol oral self-administration during adulthood, revealing sex-specific changes in ethanol intake, before and after stress.

2.2. Materials and Methods

2.2.1. Research subjects

C57BL/6J mice of both sexes (IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) were given access to housing enrichment and *ad libitum* food and water (Labdiet, 5053, PMI, Brentwood, MO). The animals were maintained on a reverse 12-hr light/12-hr dark cycle (lights “off” at 10:00 AM) and housed in a temperature- and humidity-controlled room (65-75 ^{o}F, 40-60% relative humidity). All experiments were conducted during animals’ active phase (10:00 AM – 8:00 PM). For all drinking experiments, an empty control cage was set up with two bottles that were weighed daily to account for fluid leakage due to cage and bottle handling. For all

behavioral tests, animals were habituated to the testing room and light conditions at least 30 minutes prior to the start of the test. A digital light meter was used to measure luminosity in the room for each test, reported as lux. All experiments were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

2.2.2. Maternal Drug Exposure

Female mice were habituated to being single-housed and to being exposed to one bottle of 0.2% saccharin (Sigma, St. Louis, MO) in filtered water for one week (Figure 2-1A-Habituation). After one week of habituation, female mice were observed for 30 minutes in their home cage for baseline physical signs (room: 2 lux). Signs commonly reported for morphine withdrawal were included in the analysis: jumping, wet dog shakes, head nods/shakes, teeth chattering, diarrhea, and writhing (Muldoon et al., 2014; Pinelli & Trivulzio, 1997). Female mice were then separated into an experimental (morphine + saccharin) group, referred to as “morphine dam”, and a control (saccharin) group, referred to as “control dam”. Groups were created considering the baseline physical signs, to ensure that both groups had on average a similar total number of physical signs to start. The morphine dams were given one bottle of 0.1 mg/mL free-base (f.b.) morphine (Morphine Sulfate, Spectrum Chemical MFG. Corp, New Brunswick, NJ) in 0.2% saccharin for four days and then the solution was escalated to 0.2 mg/mL f.b. morphine + saccharin for three days (Figure 2-1A). Bottles were weighed daily, and their position was alternated to avoid side preference. Morphine intake is reported as weekly averages in mg/kg/day. To confirm dependence, female mice were tested for spontaneous signs of withdrawal 8 hours after the morphine bottle was

replaced with a saccharin-only bottle, at the end of the one-week forced morphine (0.2 mg/mL) exposure. Control female mice were maintained on one bottle of 0.2% saccharin throughout this time and observed for physical signs.

The female mice were then transitioned to the continuous two-bottle choice (C2BC) phase the day after somatic signs testing (Figure 2-1A). Mice in the morphine dam group received one bottle containing 0.1 mg/mL f.b. morphine in 0.2% saccharin, and a bottle containing filtered water. Mice in the control dam group received one bottle of 0.2% saccharin solution and a bottle of filtered water. After one week of the C2BC, mice from the morphine dam group were evaluated for spontaneous physical signs of withdrawal 8 hours after removal of the morphine bottle, as described above. During week two of the C2BC paradigm, mice were evaluated again for physical signs while drug sated. Mice from the control dam group were evaluated for physical signs on the same day as the mice from the morphine dam group.

Because we were interested in investigating only the effects of maternal opioid use, female mice were placed daily in the cage of single-housed, drug-naïve males to mate for approximately five hours/day and were then returned to their home cage to continue their C2BC paradigm throughout gestation. Pregnancy was confirmed by a substantial increase in body weight after one week. The day at which pups were found is referred to as postnatal day 0 (PND 0). Offspring were allowed to lactate from their respective dam until PND 7 (Figure 2-1B). On PND 7, offspring were cross-fostered to an experienced drug-naïve dam, who had her own litter removed at the time of cross-fostering. We chose to cross-foster offspring on PND 7, because the first postnatal week is roughly equivalent to the human second trimester, with regards to brain maturation

(Barr et al., 2011; Ross et al., 2015; Semple et al., 2013). In addition, cross-fostering would allow us to evaluate early neonate outcomes after acute morphine withdrawal.

On PND 3, modified spontaneous physical signs from Barr *et al.* (2011) were recorded for 2 pups per litter (6-7 litters/ dam treatment), which included audible cries, rolling over, and full 360° body rotation. Each pup was removed from their cage and placed on a paper towel, under red light to minimize stress. Pups were observed for five minutes for spontaneous signs, and then immediately returned to their respective litter. Offspring were weighed on PND 3 and weighed every other day (odd days) thereafter until PND 15 (Figure 2-1B). Litter averages are shown for body weight data, since individual pups were not tattooed to keep track of ID. All male and female offspring cross-fostered from both control dams and morphine dams were weaned between PND 21-28 and used for neonate, adolescent, and adult testing.

2.2.3. Ultrasonic Vocalizations

Evaluation of neonate offspring ultrasonic vocalizations (USVs) from both treatment groups began on PND 2 and ended on PND 12 (Figure 2-1B). USVs were recorded on PND 2, 4, and 6 to evaluate changes related to lactation from control dams and morphine dams. USVs were also recorded on PND 8, 10, and 12 to evaluate changes associated with cross-fostering on PND 7 and potentially, morphine withdrawal, as observed in human newborns that experience NOWS. We were specifically interested in USV parameters before and after cross-fostering, so only data for PND 6 and PND 8 are reported (Figure 2-1B).

Individual pups were transferred into a small container with bedding and placed in an enclosed Styrofoam box with an ultrasonic microphone (Dodotronic Ultramic, Dodotronic, Castel Gandolfo, Italy) inserted on top. The microphone was connected to a laptop that was running Raven Pro 1.6.0 (Raven, Center for Conservation Bioacoustics, Cornell Lab of Ornithology, Ithaca, NY) to save and analyze the file as a 120-megabyte.wav file. Offspring USVs were recorded for five minutes. An average of three pups per litter was recorded on the assigned even-day PND.

USVs from each sound file were visualized using Raven software's spectrogram and were manually selected by an experimenter blinded to the treatment groups to avoid bias. The selections made were automatically incorporated into an aggregate Selection Table produced by Raven that gave various measurements for each call selected, including number of syllables, low frequency (Hz), high frequency (Hz), delta time (s), delta frequency (Hz), and center frequency (Hz). In Raven, delta time is described as the average difference between the start and end time of each call in the sound file. Delta frequency is defined as the average difference between the maximum and minimum frequency of each call in the sound file. Center frequency is defined as the average middle frequency of each call in the sound file.

We calculated the average of each measurement in the Selection Table for each pup's PND USV 5-minute file, to provide an average for a specific USV parameter for each individual pup within a litter. To compare litters across PNDs (7-9 litters per dam treatment used), the values for USV measurements for pups within a litter were averaged for a given PND based on their treatment groups, giving us a "litter average".

2.2.4. Adolescent baseline behavioral tests

Offspring were habituated to handling at least five days before adolescent testing started. Testing during adolescence occurred between PND 28 and PND 49.

To assess baseline physical signs, adolescent offspring were observed for shaking, scratching, grooming, and teeth chattering as described before (E. Perez et al., 2015; E. E. Perez & De Biasi, 2015; Quijano Cardé et al., 2021; Ramiro Salas et al., 2004, 2007, 2009). Offspring were placed in a novel cage with clean corncob bedding (room lux: 2) and observed for 20 minutes (6-9 litters examined/dam treatment). The same cages were then used for the marble burying test (MBT) to assess anxiety-like/compulsive-like behavior as previously described (Njung'e & Handley, 1991; E. E. Perez & De Biasi, 2015). Briefly, cages were filled with 5 centimeters of bedding and 20 marbles evenly spaced on top. Offspring were left undisturbed for 30 minutes (room lux: 2) and the number of marbles buried (fully buried or at least 2/3 buried) was recorded.

At least 48 hours after the MBT, offspring were tested in the Open Field Arena (OFA) test. The OFA consisted of a white plexiglass squared platform (40 centimeters by 40 centimeters) with walls (Gangitano et al., 2009; R. Salas et al., 2008; Ramiro Salas et al., 2003). The OFA was divided into a center zone (20 cm by 20 cm) and a surround zone (10 cm from wall all around OFA). The average center zone luminosity was 4 lux, while the corner surround zone luminosity was 2 lux. Offspring were placed at the center of the OFA and allowed to freely explore for 30 minutes while being recorded with ANYMAZE software (Stoeling Co, Wood Dale, IL). Locomotion and anxiety-like behavior were assessed by measuring the average total distance travelled (m) and center distance ratio (distance travelled in center zone (m)/total distance travelled (m)), respectively.

At least 48 hours after the OFA, offspring were tested in the Elevated Plus Maze (EPM), as previously described (Gangitano et al., 2009; E. E. Perez & De Biasi, 2015; R. Salas et al., 2008; Ramiro Salas et al., 2003; X. Wang et al., 2002). The luminosity used for the open arms was 4 lux, and that for the closed arms was about 1 lux. Animals were placed in the center zone of the EPM and allowed to freely explore for 10 minutes. Average time spent in the open arms (s) and open arm entry ratio (open arm entries/ open arm entries + closed arm entries) were reported to evaluate anxiety-like behavior.

At least 48 hours after the EPM, offspring were tested in the Tail Suspension Test (TST) to measure depressive-like behavior as previously described (Gangitano et al., 2009; R. Salas et al., 2008). The luminosity of the area under the tail suspension apparatus was about 4 lux. Tape was used to hold the tail onto the TST apparatus, and the animal was hung upside down for six minutes. Average time spent immobile (s) was reported.

Global Behavioral Score Classification. Six behavioral measures were used to calculate global baseline behavior scores (GBS) in offspring. The measures include: (1) physical signs, calculated as the total number of physical signs, (2) anxiety-like/compulsive-like behavior, calculated as total number of marbles buried in MBT, (3) locomotion, calculated as total distance travelled in OFA, (4) anxiety-like behavior in OFA, calculated as center distance ratio, (5) anxiety-like behavior in EPM, calculated as open arm entry ratio, (6) depressive-like behavior, calculated as total immobility time in the TST. The measures used for the GBS were chosen *a priori* based on our hypothesis that offspring from morphine-exposed dams would display increased baseline physical

signs, compulsive-like behavior, anxiety-like behavior, depressive-like behavior, and hyperlocomotion. Because our hypothesis included changes in locomotion, we used measures of anxiety-like behavior that incorporated locomotion in the measure, instead of using time in a zone (i.e. if locomotion is changed then that might influence time spent in a particular zone and affect anxiety-like measures).

As explained by O'Neal *et al*. (2020) and Quijano Cardé *et al.* (2022), z-scores were calculated for each behavioral measure. Briefly, the group mean (μ^1) for each behavioral measure was subtracted from the raw individual value (x^1) for each offspring for that behavior, and then divided by the group standard deviation, ($\boxed{x^1 - \mu^1 / \sigma^1}$) = z^1. The z-score was then multiplied by the direction (+1 or -1) for that behavioral measure, to indicate worst behavioral outcome. For example, for center distance ratio in the OFA and open arm entry ratio in the EPM, the lower the raw value, the more anxiety-like behavior the offspring displays, so the z-score for both of these behavioral measures would be multiplied by -1 to correct for the direction. Conversely, higher raw values for marbles buried indicate increased compulsive-like behavior, so the z-score is multiplied by +1 to reflect a worst behavioral outcome. Individual z-scores for each offspring were added to obtain a global behavioral score for that subject ($\boxed{\Sigma\, z^1 \ldots z^6 = \mathrm{GBS}}$). Only offspring with raw data for all behavioral measures were used for the analysis.

GBS were then used to classify offspring into 'high', 'moderate', and 'low' behavioral severity classifications based on work from O'Neal *et al.* (2020). The authors of the study found two populations that were +/- 2 standard deviations from each other where GBS scores above +1 had a worst behavioral phenotype, and scores less than -1 suggested a lower severity phenotype. Using this approach *a priori*, we classified

offspring as having a ‘high’ behavioral severity if GBS>1, ‘moderate’ if +1>GBS>-1, and ‘low’ behavioral severity if GBS<-1.

2.2.5. Adult ethanol intermittent two-bottle choice (I2BC) paradigm and restraint stress

Mice that were previously analyzed for adolescent baseline behaviors were examined for ethanol drinking behavior during adulthood using the ethanol I2BC, as previously described (Carnicella et al., 2014; Hwa et al., 2011; Quijano Cardé et al., 2021; Quijano Cardé & De Biasi, 2022). Offspring (at least 2 months of age) were habituated to being single-housed and were exposed to two 50 mL bottles of filtered water for at least one week in the home cage. Afterwards, mice were given 24-hour access to a bottle of ethanol and a bottle of water on Mondays, Wednesdays, and Fridays. On alternating days, mice were presented with two bottles containing filtered water. During week 1, or the ‘Acquisition’ phase, mice were habituated to ethanol by receiving increasing concentrations of ethanol: 3% (Monday), 6% (Wednesday), and 10% (v/v) ethanol (Friday). During weeks 2-5 of the ‘Maintenance’ phase, mice were given one bottle of 20% ethanol (v/v) and one bottle of water. Mice were then transitioned to a ‘Fading’ phase of the experiment to determine if mice would drink more to maintain the same ethanol dose they received on week 6 (20% ethanol) even when the ethanol concentration was progressively decreased in the subsequent weeks. During weeks 7-10, mice were given decreasing concentrations of ethanol each week (15%, 10%, 6%, and 3% ethanol). Presentation of the ethanol bottle occurred three hours after ‘lights off’ (1:00 PM), and 2- and 24- hour ethanol consumption (g/kg/day) and preference (%) [(ethanol ml intake/total ml fluid intake)*100] were measured. All mice

were weighed weekly. All ethanol solutions were made in filtered water (v/v) using 190-proof ethanol (Decon Laboratories Inc., King of Prussia, PA).

To investigate the effects of an acute stressor on ethanol drinking, we used the commonly used restraint stress test (Rockman et al., 1986; X. Yang et al., 2008). On week 11, mice were given one bottle of 20% ethanol and one bottle of water, to establish a pre-stress baseline. On week 12, mice were subjected to two days (Monday and Wednesday) of a 1-hour acute restraint stress in their home cage approximately 30 minutes after "lights-off". Briefly, mice were gently scruffed and guided into a plastic 50 mL conical tube with the ends cut to allow for air flow. Two hours after mice were removed from the restrainer, mice were presented with their 2BC of 20% ethanol and water, and allowed to drink for a regular ethanol session.

2.2.6. Sucrose Preference Test (SPT)

Adult mice in the ethanol I2BC paradigm were tested for anhedonia using the sucrose preference test (Falcon et al., 2016). Briefly, 48-hours after the ethanol bottle was removed, mice were given one bottle of 2.5% sucrose (v/v) in filtered water for a two-hour habituation that began roughly one hour after "lights-off". The following day, starting one hour after "lights-off" again, mice were given one bottle of 1% sucrose in filtered water and another bottle of filtered water for 8 hours. Half-way through the SPT, the bottle position was alternated. Sucrose preference (%) ((mLs sucrose intake/mLs total fluid intake)*100) was calculated.

2.2.7. Dexamethasone suppression test (DST) and blood collection

Adult mice in the ethanol I2BC paradigm that were used for SPT were also evaluated in the dexamethasone suppression test (DST), a test which has been used to test the functionality of the HPA axis (A. Moles et al., 2008). Briefly, dexamethasone 21-phosphate disodium salt (Sigma, St. Louis, MO) was dissolved in saline for a 10x stock solution. Mice were injected interperitoneally (i.p.) with 0.1 mg/kg f.b. dexamethasone, and six hours later trunk blood was collected after live decapitation, while mice were ethanol sated from the 2BC. The two bottles - 20% ethanol and water - was presented immediately after mice were injected with dexamethasone. Blood was kept on ice until centrifuged at 4°C at 10,000 RPMs for 15 minutes. Plasma was stored at -80°C for corticosterone analysis.

2.2.8. Corticosterone enzyme-linked immunoassay (ELISA)

Corticosterone levels were evaluated using an Abcam (Abcam Cat# ab108821) protocol. Briefly, samples were diluted 1:5 in 1x Diluent M. Then, samples and standards were incubated with biotinylated corticosterone protein for two hours. Wells were immediately washed five times with 1x Wash Buffer. Wells were then incubated for 30 minutes with 1x SP Conjugate and immediately washed five times. Chromogen substrate was added for 25 minutes. Then, stop solution was added and the absorbance was immediately read at 450 nm with a plate reader. Standard curves were interpolated using the sigmoidal 4PL in Prism.

2.2.9. Adult baseline behavioral tests

In a separate cohort, adult (at least two months of age) group-housed offspring from control and morphine-treated dams were habituated to handling. Physical signs, MBT, OFA, EPM, and TST were examined at least 24-hours apart. To assess baseline physical signs, adult offspring were observed for jumping, shaking, scratching, grooming, and teeth chattering. Offspring were also assessed for baseline behaviors in the MBT, OFA, EPM, and TST, like described in the previous sections.

2.2.10. Statistical analyses

The data were analyzed using Graphpad PRISM 9 and are expressed as mean +/- standard error of the mean (SEM). Litter averages are shown for neonate body weight and USV data, while individual data points are shown for dam, adolescent, and adult data. Dam and neonate outcomes were analyzed using paired t-test, t-test, or repeated measures (RM) one-way ANOVA, when appropriate. Tukey post-hoc analysis was used as recommended. Adolescent offspring data were analyzed using a two-way ANOVA to investigate the potential effect of sex and/or dam treatment, and the interaction between the two variables. GBS classifications for adolescent and adult offspring data were analyzed using an outcome versus expected chi-square test, where the control offspring percentages for each classification (‘low’, ‘moderate’, ‘high’) were used as the ‘expected’ percentages to compare to percentages obtained from offspring from morphine-exposed dams. A RM three-way ANOVA for ethanol I2BC drinking data revealed a main effect of sex, so males and females were analyzed separately using a RM two-way ANOVA with Sidak post-hoc analysis. For the I2BC experiment, the ‘Acquisition’, ‘Maintenance’, and ‘Fading’ phases were analyzed separately. For datasets

missing values at certain experimental timepoints, a mixed effects model with a Sidak post-hoc test was performed. A *p*-value of <0.05 was considered statistically significant. ROUT (Q = 1%) was used to remove significant outliers.

2.3. Results

2.3.1. Validation of a maternal morphine exposure model in mice

We developed a paradigm to model opioid use in humans and ultimately investigate the effects on offspring, by using an oral morphine self-administration protocol in female pregnant mice (Figure 2-1A). Since human mothers who are opioid-dependent begin drug use before pregnancy, we first established a paradigm where breeding-age female mice would become dependent on morphine. To create initial opioid dependence, mice were given one bottle of escalating concentrations of morphine (0.1 mg/mL – 0.2 mg/mL), which led to increased morphine intake (paired t-test; t = 5.896, df = 10, p = 0.002; Figure 2-2A). Under this treatment paradigm, female mice displayed increased spontaneous physical signs of withdrawal 8 hours after the removal of the morphine bottle, compared to their pre-treatment baseline (paired t-test; t = 6.835, df = 9, p = <0.0001; Figure 2-2B). Mice were then transitioned to a continuous two-bottle choice (C2BC) paradigm, where they received one bottle of morphine in saccharin water and one bottle of water. Based on previous 2BC morphine protocols used in the field, saccharin was added only to the morphine bottle because morphine salt is perceived as bitter and we wanted to limit the variability of the dose of morphine consumed between dams (Belknap, 1990; Belknap et al., 1993; Ferraro et al., 2005). After one week in the

morphine C2BC paradigm (week 2 of the paradigm), mice drank on average 37 mg/kg morphine solution (Figure 2-2C). Mice also displayed increased spontaneous physical signs of withdrawal 8 hours after the removal of the morphine bottle, compared to when they were morphine-sated in the C2BC (paired t-test; t = 4.315, df = 9, p = 0.0019; Figure 2-2D), and compared to control female mice that received drug-free sweetened fluid (t-test; t = 3.484, df = 20, p = 0.0023; Figure 2-2E).

Female mice were then paired with male mice while on the C2BC, until pregnancy was confirmed. A criteria of inclusion during the C2BC paradigm was for mice to drink above 10 mg/kg morphine during pregnancy, which has been shown to produce analgesia in rodents (Frances et al., 1992; Fujita-Hamabe et al., 2012). As shown in Figure 2-2C, female mice continue to drink morphine in the C2BC throughout gestation and until their offspring reach PND 7, at which point pups are cross-fostered to a drug-naïve dam. During weeks 4-6 of the C2BC paradigm, dams display slightly lower morphine intake compared to week 2 and week 3 of the paradigm (RM mixed effects analysis; F(1.748, 17.19)= 4.807, p = 0.0256; Figure 2-2C). This phenomenon could be due to being paired with the male breeder for 5 hours during the day (week 4), and also to the increased bodyweight during pregnancy (week 5 and 6). Together, our data show that morphine-exposed dams exhibit signs of dependence upon removal of the drug and continue morphine drinking during pregnancy.

2.3.2. Neonate deficits before and after cross-fostering in offspring from morphine-exposed dams

To investigate the effects of maternal morphine exposure on offspring, pups were examined during early PNDs (Figure 2-1B). PND 3 offspring that were exposed to morphine through lactation displayed increased spontaneous activity (t-test; $t = 2.527$, $df = 23$, $p = 0.0188$; Figure 2-3A). Due to limited motor function during this early developmental period, the spontaneous signs monitored included audible cries, rolling over, and full 360° body rotation (Barr et al., 2011; Zhu & Barr, 2004).

To evaluate the long-term consequences associated with early-development morphine exposure, pups were cross-fostered to a drug-naïve dam on PND 7. This allowed for offspring to experience morphine withdrawal without introducing the dam's drug-associated withdrawal behavior as a confound. Pups were weighed before and after cross-fostering, and we found an interaction between dam treatment and PND (RM mixed effects analysis; $F(6, 160)=2.884$, $p = 0.0107$), and a main effect of PND (RM mixed effects analysis; $F(1.587, 42.31)=80.41$, $p = <0.0001$) (Figure 2-3B). Interestingly, morphine offspring had a trend for decreased body weight in early PNDs, compared to control offspring.

To evaluate distress that might be comparable to high-pitched crying seen in newborns with NOWS, ultrasonic vocalizations (USVs) were recorded in mice offspring before and after cross-fostering. We were specifically interested in evaluating USVs at PND 6 and PND 8, which corresponds to timepoints right before and after cross-fostering, respectively. This approach was chosen to evaluate changes while the offspring were lactating from morphine-treated dams (PND 6) and when they would potentially be undergoing acute drug withdrawal (PND 8) since they could no longer

lactate from their respective dam. Offspring from morphine-exposed dams displayed no changes in the number of calls compared to control offspring (Figure 2-3C). There was also no significant effect of dam treatment on delta time (i.e. length) of calls, but there was a main effect of PND (RM two-way ANOVA; $F(1, 14)=18.22$, $p = 0.0008$; Figure 2-3D). Similarly, there was no effect of dam treatment on the frequency range (delta frequency) of calls, but there was a main effect of PND (RM two-way ANOVA; $F(1, 14)=39.70$, $p = <0.0001$; Figure 2-3E).

However, there was a significant main effect of dam treatment on the frequency parameters of offspring's USVs (Figure 2-3F-H). Offspring from morphine-exposed dams had calls of higher center frequency (RM two-way ANOVA; $F(1, 14)=6.304$, $p = 0.0249$; Figure 2-3F) compared to control offspring, and post-hoc analysis revealed that this change was statistically significant after cross-fostering (PND 8). Offspring from morphine-exposed dams also had calls of higher low-frequency points (RM two-way ANOVA; $F(1, 14)=5.696$, $p = 0.0317$; Figure 2-3G). In addition, offspring exposed to prenatal-perinatal morphine had higher high-frequency points in the calls (RM two-way ANOVA; $F(1, 14)=4.713$, $p = 0.0476$; Figure 2-3H), and there was a main effect of PND (RM two-way ANOVA; $F(1, 14)=14.08$, $p = 0.0021$; Figure 2-3H). Post-hoc analysis revealed that offspring from morphine-exposed dams had higher high-frequency points in their calls both before cross-fostering (PND 6) and after cross-fostering (PND 8), compared to control offspring. Overall, these results show that maternal morphine exposure alters neonatal spontaneous activity, body weight, and ultrasonic vocalization acoustic parameters.

2.3.3. Changes in anxiety-like/compulsive-like behavior in adolescent offspring from morphine-exposed dams

Offspring from morphine-exposed dams were evaluated for changes in behavior during adolescence to further understand the consequences of maternal opioid exposure during a critical period of development (Figure 2-4A). We used various behavioral tests to assess baseline changes in somatic and affective behavior, including measures to investigate locomotion, compulsive-like, anxiety-like, and depressive-like behavior. Behavioral measures were assessed for an effect of dam treatment and/or sex, but because no effect of sex was observed, males and females were combined. There was no difference in baseline physical signs between offspring from morphine-exposed dams and control offspring (Figure 2-4B). However, offspring from morphine-exposed dams buried more marbles than offspring from control dams in the marble burying test (MBT) (t-test; t =2.971, df = 69, p=0.0041; Figure 2-4C), displaying more anxiety-like/compulsive-like behavior.

There was no effect of dam treatment in the Open Field Arena (OFA) for total distance travelled or center distance ratio (Figure 2-4D-E), nor did we detect significant differences in the open arm entry ratio in the Elevated Plus Maze (EPM; Figure 2-4F). Similarly, when offspring were assessed for depressive-like behavior in the Tail Suspension Test (TST), no significant difference in total immobility time was observed (Figure 2-4G).

Although there were no significant differences in behavior when individual tests were considered, we were interested in integrating multiple behavioral outcomes into a composite score. This would allow us to characterize offspring behavior holistically, which has been used in multiple areas of research (El-Kordi et al., 2013; Guyenet et al.,

2010; Möller et al., 2018; O'Neal et al., 2020; Pereira de Souza Goldim et al., 2020; Shahi et al., 2019). The use of a global severity score classification system allows us to examine the distribution of animals' performance across multiple behavioral tests, where higher values represent higher behavioral symptom severity. As shown in Figure 2-4H, offspring from morphine-exposed dams have similar global behavioral scores (GBS) compared to offspring from control dams. To determine the distribution of adolescent offspring GBS, experimental scores were characterized into 'high' (GBS>1), 'moderate' (-1<GBS<1), and 'low' (GBS<-1) phenotypes. Because there was a trend ($p=0.0898$) for a main effect of dam treatment when the three GBS classifications were evaluated for the offspring (data not shown), we evaluated the percentage of offspring in each GBS classification. Among control offspring, 39% were classified as having a 'low' GBS phenotype, 29% were 'moderate', and 32% were 'high' (Figure 2-4I). However, offspring from morphine-exposed dams had a higher percentage of 'high scores' (41%), and 35% classified as having a 'low' severity phenotype, while 24% were 'moderate' (Figure 2-4I). In addition, when sex was investigated, 46%, 23%, and 31% of male control offspring fell under the 'low', 'moderate', and 'high' classification, respectively (Figure 2-4J). Male offspring from morphine-exposed dams were characterized at similar percentages in each GBS classification (low=50%, moderate=29%, and high=21%) (Figure 2-4H). Conversely, female offspring from morphine-exposed dams, had a trend for a higher percentage being classified in the 'high' category (60%), compared to control female offspring (33%) (Figure 2-4K). Twenty percent of female offspring from morphine-exposed dams were categorized as 'moderate' and 'low' scorers based on their GBS, while 33%-34% of control female offspring were categorized as 'moderate' and 'low' (Figure 2-4K). Although the GBS classification is not significantly different between offspring, the higher percent of 'high' GBS phenotype in the offspring from morphine-

exposed dams is intriguing in that it suggests that early life morphine exposure might lead to an increase in the number of offspring that have a more severe phenotype when considering a broad array of behaviors, as opposed to very significant deficits in any one behavioral measure.

2.3.4. Changes in baseline behavior in adult offspring from morphine-exposed dams

We were interested in the possibility that the behavioral phenotypes we observed could persist beyond adolescence and into adulthood. Therefore, in a separate cohort of control and morphine-exposed offspring, we evaluated baseline adult behavior to determine the long-term effects of maternal morphine exposure using the same battery of behavioral tests used for adolescent mice (Figure 2-5A). Behavioral measures were assessed for an effect of dam treatment and/or sex, but when no effect of sex was detected, males and females were combined. Offspring from morphine dams did not significantly differ from control offspring in baseline total physical signs (Figure 2-5B), compulsive-like behavior in the MBT (Figure 2-5C), locomotion or anxiety-like behavior in the OFA (Figure 2-5D-E), and depressive-like behavior in the TST (Figure 2-5H). However, when adult offspring were evaluated for anxiety-like behavior in the EPM, offspring from morphine-exposed dams displayed no difference in entry ratio (Figure 2-5G), but did display decreased time spent in the open arms, compared to control offspring (t-test; $t = 2.935$, $df = 35$, $p = 0.0059$; Figure 2-5F). This suggests that offspring from morphine-exposed dams have increased anxiety-like behavior in adulthood, when considering time in the open arm.

We also used the GBS to integrate the multiple behavioral outcomes into a composite score which allowed us to characterize adult offspring behavior holistically, as described above. Although there was no difference in overall global behavioral score between offspring from morphine-exposed dams and control dams (Figure 2-5I), the percentage of offspring that fell into each GBS classification was of interest. For control offspring, 48% were classified as having a 'low' severity phenotype, 26% were 'moderate', and 26% were 'high' (Figure 2-5J). However, among offspring from morphine-exposed dams, only 28% were classified as 'low', 33% were 'moderate', and 39% were 'high' (Figure 2-5J), suggesting that a higher percentage of offspring from morphine-exposed dams might be more behaviorally vulnerable. Although the sample size was small, Figure 2-5K shows that the percentage of male offspring from morphine-exposed dams in each GBS classification was different than that of male control offspring (chi-square test; DF=2; p =0.0052). Among male control offspring, 70% were classified as having a 'low' GBS severity phenotype, 10% were 'moderate', and 20% were 'high' (Figure 2-5K). However, among male offspring from morphine-exposed dams, 22% were 'low', 33% were 'moderate', and 45% were 'high' (Figure 2-5K). Conversely, female offspring from morphine-exposed dams had a similar percent of offspring that fell into the three GBS classifications when compared to female control offspring (Figure 2-5L). For example, 33% of mice were classified as having a 'high' GBS severity phenotype in both groups.

Together, our results suggest that maternal morphine exposure might have subtle, long-term consequences throughout the offspring's life span, as reflected by changes that persist in adulthood. Offspring from morphine-exposed dams display increased baseline levels of anxiety-like behavior during adulthood. In addition, a much

higher percent of male offspring from morphine-exposed dams fall into the 'high' and 'moderate' GBS severity classification. This suggests that not only are specific phenotypes altered by prenatal-perinatal opioid exposure, but that, overall, male offspring are at risk of developing more severe behavioral phenotypes during adulthood, a phenomenon that could be revealed or exacerbated by stress or drug use.

3.3.5. Male offspring from morphine-exposed dams display decreased two-hour ethanol intake

Given the well-documented interactions between alcohol and the opioid system (Arias & Kranzler, 2008; Berrettini, 2013; Gianoulakis, 2001; Gianoulakis et al., 1989; Job et al., 2007; Oslin et al., 2006), we next wanted to assess alcohol use risk in offspring maternally exposed to morphine. The offspring tested in the battery of behavioral tests during adolescence were allowed to mature into adulthood and were then evaluated in an ethanol intermittent two-bottle choice (I2BC) paradigm (Figure 2-6A), which has been used to assess voluntary ethanol intake (Carnicella et al., 2014; Hwa et al., 2011; Quijano Cardé et al., 2021; Quijano Cardé & De Biasi, 2022). A three-way ANOVA revealed a main effect of sex where female mice (regardless of treatment) drank significantly more ethanol than male mice, so data and analyses are presented separately for each sex. Alcohol-related behaviors were evaluated at three different phases of the I2BC – Acquisition, Maintenance, and Fading - for both male and female offspring.

Acquisition phase of I2BC

Ethanol drinking patterns were first evaluated for the 'Acquisition' phase of the I2BC, where mice were given increasing concentrations of ethanol during the first week of exposure. Figure 2-6B shows a significant main effect of concentration for 2-hour intake during the 'Acquisition' phase for male offspring (RM mixed effects analysis; $F(1.417, 34.02) = 133.9$, $p < 0.0001$) and a main effect of dam treatment (RM mixed effects analysis; $F(1, 26) = 6.176$, $p=0.0197$). Specifically, post-hoc analysis revealed that male offspring from morphine-exposed dams drink less ethanol (g/kg) at the 6% concentration during the first two hours of the session, compared to male control offspring. When ethanol intake (g/kg) was evaluated during the 24-hour sessions of the 'Acquisition' phase (Supplemental Figure 2-1A), although not significant, a trend ($p=0.0791$) for an effect of dam treatment was present for male offspring. There was a significant main effect of concentration for the 24-hour ethanol intake (RM two-way ANOVA; $F(1.648, 42.86) = 138.9$, $p < 0.0001$; Supplemental Figure 2-1A). With regards to male offspring's 2-hour ethanol preference (%) during the 'Acquisition' phase (Figure 2-6C), there was a main effect of concentration (RM mixed effects analysis; $F(1.801, 43.22) = 8.540$, $p < 0.0001$), but no main effect of dam treatment. In addition, there was no significant main effect or interaction of concentration and/or dam treatment during male offspring's 24-hour ethanol preference during the 'Acquisition' phase (Supplemental Figure 2-1B).

In female offspring, the 2-hour session for the 'Acquisition' phase of the I2BC revealed a significant main effect of concentration for ethanol intake (RM mixed effects analysis; $F(1.770, 35.39) = 134.3$, $p < 0.0001$; Figure 2-6D) and preference (RM mixed effects analysis; $F(1.621, 32.42) = 7.813$, $p = 0.0030$; Figure 2-6E), but no effect of dam treatment. Similarly, for female offspring's 24-hour ethanol intake (Supplemental Figure

2-1C) there was a main effect of concentration (RM two-way ANOVA; $F(1.818, 40.00) = 175.6$, $p < 0.0001$), but no significant effect of dam treatment. Although not significant, there was a trend (p=0.0699) for a main effect of concentration, but no significant effect of dam treatment on female offspring's 24-hour ethanol preference for the 'Acquisition' phase (Supplemental Figure 2-1D).

Together, this reveals that male -but not female- offspring from morphine-exposed dams drink lower amounts of ethanol in the I2BC, but have no changes in ethanol preference.

Maintenance phase of I2BC

Ethanol drinking patterns were next evaluated during the 'Maintenance' phase of the I2BC, where mice were exposed every other day for four weeks (weeks 2-5) to two bottles, one containing 20% ethanol and the other containing water. In male offspring during the 'Maintenance' phase, there was no effect of week, and although not significant, a trend (p=0.0793) was present for dam treatment for two-hour ethanol intake (g/kg) (Figure 2-6B). In addition, there was no effect of week or dam treatment for males' 24-hour ethanol intake during the 'Maintenance' phase (Supplemental Figure 2-1A). With regards to male offspring's ethanol preference during the 'Maintenance' phase, there was a main effect of week for the two-hour session (RM two-way ANOVA; $F(2.163, 56.23) = 3.369$, $p= 0.0380$; Figure 2-6C) and 24-hour session (RM two-way ANOVA; $F(1.900, 49.40) = 6.162$, $p= 0.0047$; Supplemental Figure 2-1B), but no main effect of dam treatment.

Female offspring did not show a significant difference of week or dam treatment for 2-hour (Figure 2-6D) and 24-hour (Supplemental Figure 2-1C) ethanol intake during the 'Maintenance' phase of the I2BC. However, there was a main effect of week for both 2-hour (RM mixed effects analysis; $F(2.746, 59.50) = 4.141$, $p = 0.0118$; Figure 2-6E) and 24-hour ethanol preference (RM two-way ANOVA; $F(2.848, 62.66) = 6.698$, $p = 0.0007$; Supplemental Figure 2-1D), but no significant effect of dam treatment.

Together, our data show that ethanol intake and preference during the 20% ethanol 'Maintenance' phase of the I2BC are similar to control in both male and female offspring from morphine-exposed dams, implying that there is no effect of dam treatment.

Fading phase of I2BC

Lastly, ethanol drinking patterns were evaluated for the 'Fading' phase of the I2BC, where mice were given decreasing concentrations of ethanol (20%, 15%, 10%, 6%, 3%) for the remaining five weeks of the paradigm. As shown in Figure 2-6B, analysis of the 2-hour ethanol intake during the 'Fading' phase for male offspring revealed a significant main effect of concentration (RM two-way ANOVA; $F(1.960, 50.95) = 79.10$, $p < 0.0001$), a main effect of dam treatment (RM two-way ANOVA; $F(1, 26) = 4.267$, $p=0.0490$), and an interaction between concentration and dam treatment (RM two-way ANOVA; $F(4, 104) = 3.079$, $p=0.0193$). Specifically, our data suggest that male offspring from morphine-exposed dams consume less ethanol at various concentrations during the 2-hour 'Fading phase' of the paradigm. When the 24-hour ethanol intake during the 'Fading' phase was evaluated, there was a significant main effect of

concentration (RM two-way ANOVA; $F(2.562, 66.62) = 142.4$, $p < 0.0001$; Supplemental Figure 2-1A), but not dam treatment. With regards to male offspring's 2-hour ethanol preference during the 'Fading' phase, there was a main effect of concentration (RM two-way ANOVA; $F(2.335, 60.71) = 11.19$, $p < 0.0001$; Figure 2-6C), but no significant main effect of dam treatment and a trend for an interaction (p=0.0927). Similarly, during male offspring's 24-hour ethanol preference in the 'Fading' phase, there was a main effect of concentration (RM two-way ANOVA; $F(2.155, 56.04) = 84.08$, $p < 0.0001$; Supplemental Figure 2-1B), but no main effect of dam treatment.

No differences were detected when comparing control and morphine-exposed female offspring. We found a significant main effect of concentration for ethanol intake (RM two-way ANOVA; $F(2.219, 48.81) = 72.56$, $p < 0.0001$; Figure 2-6D) and preference (RM two-way ANOVA; $F(2.664, 58.62) = 24.01$, $p < 0.0001$; Figure 2-6E) at the 2-hour timepoint during the 'Fading' phase of the I2BC but no effect of dam treatment. Similarly, at 24-hour there was a significant main effect of concentration for ethanol intake (RM two-way ANOVA; $F(2.049, 45.07) = 108.6$, $p < 0.0001$; Supplemental Figure 2-1C) and preference (RM two-way ANOVA; $F(3.099, 68.19) = 144.0$, $p < 0.0001$; Supplemental Figure 2-1D), but no effect of dam treatment.

Overall, our results indicate that male -but not female- offspring from morphine-exposed dams drink lower amounts of ethanol during the initial 2-hour phase of the 'Fading' experiment although there are no changes in ethanol preference.

It should be noted that there was a significant main effect of dam treatment (RM two-way ANOVA; $F(1,26) = 7.678$, $p = 0.0102$; Supplemental Figure 2-2B) for 24-hour

total fluid intake for male offspring, and a main effect of week (RM two-way ANOVA; $F(2.576,66.98) = 18.00$, $p = <0.0001$), where male offspring from morphine-exposed dams had lower total fluid intake compared to male control offspring. This main effect of dam treatment for total fluid intake was not observed at the two-hour timepoint (Supplemental Figure 2-2A, 2-2C).

2.3.6. Acute stress decreases ethanol intake and preference in male offspring from morphine-exposed dams

We next set-out to examine the effects of stress on subsequent ethanol drinking behavior in offspring exposed to prenatal-perinatal morphine. Preclinical studies have shown that offspring exposed to *in utero* morphine display behavioral changes after acute stressors, due to dysregulation of HPA axis hormone regulation and functionality (Rimanóczy et al., 2003; Šlamberová et al., 2004; Klausz et al., 2011b; Laborie et al., 2005), but none have examined the effects of stress on ethanol intake and preference in the I2BC paradigm.

After the 'fading' phase of the ethanol I2BC paradigm, offspring were given a 2BC between 20% ethanol and water for one week (week 11) to establish a pre-stress baseline for ethanol intake and preference. While in ethanol withdrawal (~21 hours after ethanol was removed), offspring were placed in a restraint stress in their home-cage for an hour. After this, mice were allowed to recover for an hour before being presented with their ethanol 2BC between 20% ethanol and water. Offspring were placed in the restraint stressor, like described above, on a second day to investigate whether the offspring habituate to the stressor.

There was a main effect of time (RM two-way ANOVA; $F(2.618,47.12) = 47.63$, $p = <0.0001$; Figure 2-7A), where both male control offspring and male offspring from morphine-exposed dams had lower 2-hour ethanol intake on the first and second day of stress, but had higher ethanol intake on the post-stress day, compared to their own week 11 pre-stress baseline intake. There was also a main effect of time (RM two-way ANOVA; $F(2.814,50.66) = 49.78$, $p = <0.0001$; Figure 2-7B) for 2-hour ethanol preference, where both male control and male offspring from morphine-exposed dams had lower preference after the first and second day of stress, compared to their own pre-stress baseline.

There was also a significant main effect of time (RM two-way ANOVA; $F(1.977,35.58) = 22.31$, $p = <0.0001$; Figure 2-7C) for 24-hour ethanol intake. Specifically, male control offspring display lower 24-hour ethanol intake after the first day of the stressor, compared to their pre-stress baseline. Furthermore, on the second day of the restraint stress, male control offspring return to ethanol consumption levels comparable to their pre-stress baseline. Although male offspring from morphine-exposed dams also display reduced 24-hour intake on the first day of the stressor, they interestingly show lower ethanol intake on the second day of the stressor, suggesting an inability to habituate to subsequent stress. With regards to 24-hour ethanol preference, there was a main effect of time (RM two-way ANOVA; $F(1.947,35.05) = 24.54$, $p = <0.0001$; Figure 2-7D), where both male control and male morphine-exposed offspring had lower preference after the first day of stress, compared to their own pre-stress baseline.

A significant main effect of time (RM two-way ANOVA; $F(2.200,33.00) = 8.682$, $p = 0.0007$; Figure 2-7A) was also observed for 2-hour ethanol intake, where specifically

female control offspring displayed higher 2-hour ethanol intake on the post-stress day compared to their pre-stress baseline intake. There was also a main effect of time (RM two-way ANOVA; $F(2.165,32.47) = 8.530$, $p = 0.0008$; Figure 2-7B) for 2-hour ethanol preference, where female morphine-exposed offspring had lower preference after the second day of stress, compared to their own pre-stress baseline, and female control offspring displayed a similar trend (p=0.0939). A trend for a main effect of time (p=0.0508) and a trend for an interaction between dam treatment and time (p=0.0577) were found for female offspring's 24-hour ethanol intake (Figure 2-7C). There was also a significant main effect of time (RM two-way ANOVA; $F(1.916,28.74) = 4.522$, $p = 0.0208$; Figure 2-7D) for 24-hour ethanol preference, where there was a trend for female morphine-exposed offspring to display lower preference after the first (p=0.0875) and second (p=0.0568) day of stress, compared to their own pre-stress baseline.

Although there was no significant main effect of dam treatment, the data above suggest that an acute stressor affects both control and morphine-exposed dam offspring's 2-hour and 24-hour drinking behavior, in a sex-dependent manner. Interestingly, although male control offspring were able to habituate to the second day of the same stressor, male offspring from morphine-exposed dams did not, and continued to display lower 24-hour ethanol intake.

Preliminary findings suggest that the decrease in ethanol intake at baseline during the I2BC paradigm, and after the second day of an acute restraint stress, was not due to anhedonia-like behavior, measured by the sucrose preference test (Supplemental Figure 2-3A), or impairments in the HPA axis negative feedback regulation, measured by corticosterone levels after the dexamethasone-suppression test (Supplemental Figure 2-3B).

2.4. Discussion

Our maternal morphine C2BC paradigm demonstrated that morphine dams display signs of dependence and voluntarily drink morphine throughout gestation. Maternal morphine exposure with this paradigm increases neonate spontaneous activity, decreases body weight before cross-fostering, and alters various USV frequency parameters. The set of experiments presented in this study also demonstrates subtle sex-specific alterations in adolescent and adult offspring exposed to pre- and perinatal morphine. Overall, the data presented supports the hypothesis that maternal opioid exposure produces subtle alterations in offspring behavior throughout development.

Our study is one of few to investigate offspring outcomes using a maternal oral self-administration model that starts before gestation, continues throughout gestation, and extends one week postnatally. Most preclinical studies investigating the effects of *in utero* morphine exposure on offspring behavior have used daily injections or forced oral solution, making it difficult to discern whether the effect seen in offspring is due to an interaction of maternal stress with opioid exposure, or solely due to opioid exposure (Chiou et al., 2003; Glick et al., 1977; Klausz et al., 2011; Nasiraei-Moghadam et al., 2013; Siddiqui et al., 1997; Sobor et al., 2010; Timár et al., 2010; P. L. Wu et al., 2018; S. N. Yang et al., 2003). In addition, the duration of maternal opioid administration varies across studies. For example, studies differ among each other for using a partial gestation, full gestation, or gestation and lactation maternal opioid paradigm (Chiou et al., 2003; De Vries et al., 1991; Dutriez-Casteloot et al., 1999; Eriksson & Rönnbäck, 1989; Gagin et al., 1997; Glick et al., 1977; Jóhannesson & Becker, 1972; Klausz et al.,

2011; Laborie et al., 2005; Lesage et al., 2000; Ramsey et al., 1993; Rimanóczy et al., 2003; Sarkaki et al., 2008; Schindler et al., 2004; Shen et al., 2016; Siddiqui et al., 1997; Sobor et al., 2010; Tan et al., 2015; Timár et al., 2010; Wagner et al., 1986; P. L. Wu et al., 2018; S. N. Yang et al., 2003). Each of these exposure paradigms models specific critical developmental periods for the fetus and can confer paradigm-specific behavioral alterations.

An extended maternal morphine treatment that continues throughout the lactation period might be necessary to recapitulate the clinical outcomes of prenatal morphine and NOWS, considering that rodent gestation/early offspring postnatal period has been compared to late human gestation and newborn birth, when considering various morphological and functional milestones relating to eye, cardiac, immune, and brain development (Clancy et al., 2001; Craig et al., 2003; Holsapple et al., 2003; Krishnan et al., 2014; Kroon et al., 2019; Lazic, 2012; Rice & Barone, 2000; Richard & Flamant, 2018; Van Cruchten et al., 2017). Due to the short gestation period compared to humans, many developmental processes (e.g. myelination and immune function) in rodents continue postnatally (Craig et al., 2003; Holsapple et al., 2003). Caution is therefore warranted when making direct developmental comparisons between species since this is dependent on the processes being investigated and the developmental window studied. Overall, early rodent postnatal days might be an important consideration when developing maternal drug exposure paradigms. For this reason, we cross-fostered the offspring at PND 7 to a drug-naïve dam rather than removing the morphine bottle from the dam, thereby preventing maternal withdrawal behavior from becoming a confound in the study. We also wanted the rodent pups to undergo morphine withdrawal and potentially experience characteristics of NOWS that can be

investigated before weaning and might produce long-term behavioral consequences. Most clinical and preclinical studies have shown that offspring from opioid-dependent mothers display either reduced body weight or no change in body weight (Corr et al., 2018; Dutriez-Casteloot et al., 1999; Gagin et al., 1997; Jones et al., 2010; Kaltenbach et al., 2018; Klausz et al., 2011; Laborie et al., 2005; Ramsey et al., 1993; Shen et al., 2016; Siddiqui et al., 1997; Siu & Robinson, 2014; Timár et al., 2010). However, some preclinical studies like Chiang *et al*. (2010) and Timar *et al.* (2010) have reported increased body weight in PND 7, PND 14, and PND 21 offspring after maternal morphine exposure. Although we anticipated decreased body weight in offspring from morphine-exposed dams even after cross-fostering, our data suggest that cross-fostering might stunt growth in control offspring and/or increase overall pup mortality (data not shown for pup mortality – main effect of cross-fostering). This phenomenon has also been reported in other models of cross-fostering (Santangeli et al., 2016). Cross-fostering has been shown to affect both maternal and offspring behavior (Dulor Finkler et al., 2020; Gauthier et al., 2015; R. Šlamberová et al., 2010; I. Vathy et al., 2007), likely due to the stress associated with the new environment and alterations in the mother-infant relationship. This posits the question of whether the drug-naïve dam euthanized the most “vulnerable” offspring, and whether the subtle behavioral effect we observe between control and morphine exposed offspring might be due to the fact that we tested the “resilient” offspring that survived after cross-fostering.

Preclinical studies aim to model maternal opioid exposure that results in offspring outcomes comparable to those of human newborns experiencing NOWS. For example, studies have examined developmental milestones in rodents, as well as pup mortality and bodyweight and compared them to newborn outcomes in clinical studies (Chiang et

al., 2010; Dutriez-Casteloot et al., 1999; Eriksson & Rönnbäck, 1989; Gagin et al., 1997; Jóhannesson & Becker, 1972; Laborie et al., 2005; Ramsey et al., 1993; Siddiqui et al., 1997; Sobor et al., 2010; Timár et al., 2010). To our knowledge, this is the first study to investigate how maternal morphine exposure alters ultrasonic vocalizations in pups, as a correlate to high-pitched crying in human newborns and as a characteristic of NOWS. Similar to our results, one study found that offspring from oxycodone-exposed dams have higher frequency USVs than control offspring (Zanni et al., 2021). Another study also found that neonate offspring injected with morphine from PND 1 – 14 had increased USV frequency parameters (Borrelli et al., 2021). Various studies and reviews have focused on understanding vocalizations and changes in their acoustic parameters, including how upward shifts in frequency modulation is usually associated with an increase in infant distress (Brudzynski, 2015; Castellucci et al., 2019; Esposito et al., 2013; Hahn & Lavooy, 2005; Kromkhun et al., 2013; Lingle et al., 2012; Parga et al., 2020; Wasz-Höckert et al., 1985). Although our work did not further probe the specific brain-regions and mechanisms that lead to alterations in USV frequency parameters in offspring from morphine-exposed dams, other studies have shown that the periaqueductal grey (PAG), a brain region with dense expression of opioid receptors, and the opioid receptor system, are important for USV syllable production (D'Amato, 2021; Goodwin & Barr, 2005; Tschida et al., 2019). For example, PAG lesions decrease USVs in pups (Wiedenmayer et al., 2000) and mu-opioid receptor knockout (*Orpm*$^{-/-}$) pups emit less USV calls compared to their littermates in response to maternal isolation (Anna Moles et al., 2004). Offspring exposed to opioids *in utero* display changes in the opioid receptor system (Chiou et al., 2003; Ilona Vathy et al., 2003), which further supports our finding that offspring from morphine-exposed dams have profound changes in USV-related parameters. The functional role of brain-region specific changes in opioid

receptor and endogenous opioid levels in areas such as the PAG merits further investigation.

In addition to changes in neonatal outcomes, offspring from morphine-exposed dams also display changes in baseline behavior during adolescence and adulthood. Although we found no significant differences in locomotion or depressive-like behavior, adolescent offspring from morphine-exposed dams displayed increased anxiety-like/compulsive-like behavior in the MBT. During adulthood, offspring from morphine dams displayed increased anxiety-like behavior in the EPM. Contrary to what we found, a few preclinical studies investigating the effects of prenatal morphine exposure found either decreased anxiety-like behavior or no changes in anxiety-related behaviors, which highlights how duration and dose of maternal morphine exposure can have seemingly opposite effects in offspring (Klausz et al., 2011; Tan et al., 2015). Interestingly, similar to our results of increased anxiety-like/compulsive-like behavior in offspring from morphine-exposed dams, male offspring from morphine-exposed parents displayed decreased percent open arm time in the EPM (Sabzevari et al., 2019; Vousooghi et al., 2018), increased grooming, and increased marbles buried (Rohbani et al., 2019), suggesting increased anxiety-like/compulsive-like behavior. Similarly, male offspring from prenatally morphine-exposed dams displayed increased number of visited squares alongside the OFA walls, indicative of increased anxiety-like behavior, while ovariectomized females did not show differences in the OFA, regardless of prenatal treatment (Romana Šlamberová et al., 2002). Together with our results, these data suggest increased behavioral vulnerability in male offspring from morphine-exposed dams, while there are no apparent changes in female offspring behavior.

Prenatal or early life stress can increase susceptibility to various behavioral manifestations in male rodents later in adulthood (Columba-Cabezas et al., 2009; Lebow et al., 2019; Sarkar, 2015). In this context, prenatal opioid exposure and the experience of NOWS might also be viewed as a stressor capable of modifying behavior later in life. One potential explanation for observing changes in adulthood - and not adolescence - in male offspring could be that a more severe behavioral phenotype is unmasked among male offspring from morphine-exposed dams once all hormonal, chemical, and circuitry-related changes have matured in adulthood (Sinclair et al., 2014). Interestingly, a higher percentage (45% vs. 20% in control offspring) of male offspring from morphine-exposed dams were classified as having higher and more severe global behavioral scores during adulthood. Merhar *et al*. (2019) reported that 40% of opioid-exposed newborns exhibit significant brain alterations, suggesting that the disruption of key processes during the development of the nervous system might increase vulnerability to behavioral deficits later in life. Similar to the percentage value in the clinical data, our study finds that 39% of adolescent offspring and 41% of adult offspring from morphine-exposed dams fall under the 'High' GBS classification, suggesting a more severe behavioral phenotype. Analyzing offspring behavior using a composite score might therefore help to identify vulnerable sub-populations of individuals that need additional non-pharmacological and/or pharmacological interventions. At a minimum, such stratification might improve testing drug efficacy, as proposed by the Food and Drug Administration (FDA, 2019).

Offspring exposed prenatally to morphine have altered sensitivity to drugs, including morphine, cocaine, and methamphetamine, and display changes in drug-reward related behavior (Akbarabadi et al., 2018; Chiang et al., 2014; Gagin et al., 1997; Glick et al., 1977; He et al., 2010; Jiang et al., 2011; Ramsey et al., 1993; Sadat-Shirazi

et al., 2019; Shen et al., 2016; Timár et al., 2010; Y. Wang et al., 2017; L. Y. Wu et al., 2009). Nygaard *et al*. (2020) showed that although there were no significant differences in alcohol consumption in a one-year span in adults whose mothers misused heroin, a significantly higher proportion of those individuals reported misusing alcohol during their lives. To date, no other preclinical study has established a relationship between *in utero* morphine exposure and offspring alcohol use, and the interaction of both of these with an acute stressor. Although we hypothesized that offspring exposed to pre- and perinatal morphine would have higher ethanol intake and preference, we surprisingly found that male offspring from morphine-exposed dams had lower 2-hour ethanol intake, compared to male control offspring, despite no changes in alcohol preference. 'Front-loading' behavior, wherein the largest amount of ethanol consumed is observed toward the onset of EtOH access, is thought to reflect increased motivation to experience the rewarding effects of ethanol (Darevsky et al., 2019; Linsenbardt & Boehm, 2014; Rhodes et al., 2007; Salling et al., 2018; Wilcox et al., 2014), and, therefore, it is tempting to speculate that early exposure to morphine changes the subjective reward to ethanol. Among other mechanisms, alcohol leads to hypothalamic activation and increased levels of glucocorticoids which modify reward-related behaviors by stimulating mesencephalic dopaminergic transmission and increasing norepinephrine (NE) levels in the prefrontal cortex (PFC) (Piazza & Le Moal, 1997). Reduced ethanol drinking in morphine-exposed male offspring at the 2-hour timepoint before and after an acute restraint stress might be due to hypoactivity of the stress response and/or hypothalamic-pituitary-adrenal (HPA) axis, which has been shown to be dysregulated in rodent offspring exposed to *in utero* morphine (Klausz et al., 2011; Laborie et al., 2005; Rimanóczy et al., 2003; Romana Šlamberová et al., 2004). Further studies are needed to understand the influence of maternal morphine exposure on HPA axis function, and consequently the effects on

ethanol use. It is still unclear how alterations in fetal development by gestational opioids compound with other factors, such as chronic unpredictable stress or subsequent drug exposure, manifest in adulthood.

2.5. Conclusion

The data presented supports the hypothesis that prenatal-perinatal morphine exposure alters offspring behavior. Although not modeled in our study, important factors that influence and can exacerbate human offspring outcomes include: mother's poly-drug use, socioeconomic stress experienced by pregnant mothers, and stressors experienced by the offspring. Questions left to be answered include whether or not stress during adolescence and/or adulthood can "push" offspring exposed prenatally to opioids from the 'moderate' behavioral severity phenotype to the 'high' category, and whether chronic stress can alter adolescent and adult drug reward sensitivity, including commonly used drugs like ethanol and tobacco products.

2.6. Figures

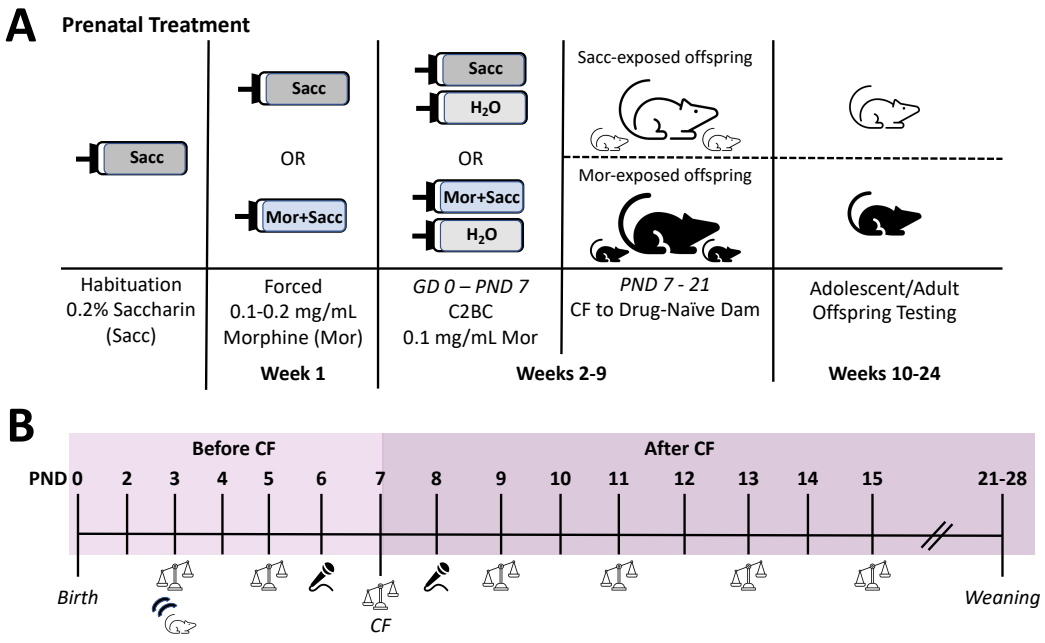


Figure 2-1: Experimental schemes for maternal morphine exposure and offspring behavioral evaluation.

(A) After a week of habituation to 0.2% saccharin, female mice drank from a single bottle containing either 0.2% saccharin or 0.2 % saccharin + morphine. Mice were then transitioned to a C2BC paradigm that lasted throughout mating, gestation, and the first week after delivery. On PND 7, offspring were cross-fostered (CF) to drug-naïve dams and were subsequently tested in various behavioral paradigms during both adolescence and adulthood. (**B**) Evaluation of offspring behavior prior to weaning (PND 0 – 28) was conducted before and after cross-fostering and included observation of spontaneous activity (moving mouse icon) on PND 3, recording of ultrasonic vocalizations (microphone icon) on PND 6 and PND 8, and measurement of body weight (scale icon) on PND 3, 5, 7, 9, 11, 13, 15.

GD = Gestation Day; Mor= Morphine; Sacc= Saccharin; H_2O= Water; PND= Postnatal Day; C2BC= Continuous Two-Bottle Choice

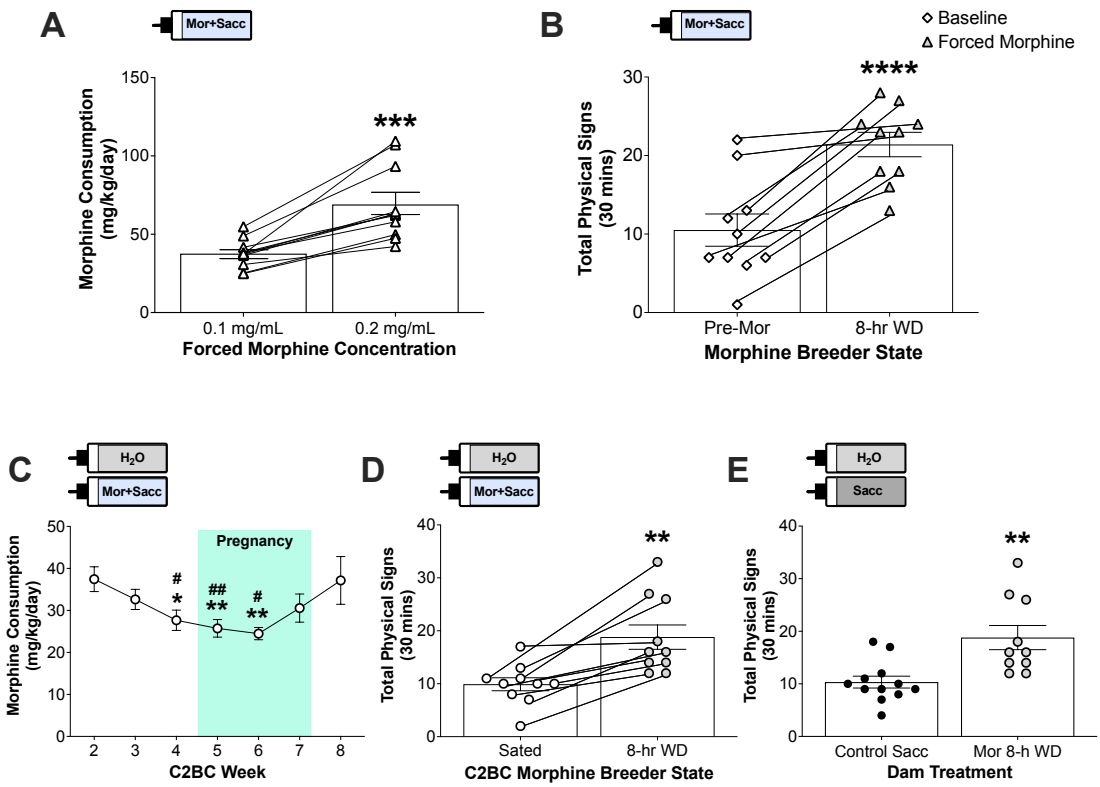


Figure 2-2: Validation of the paradigm for maternal morphine exposure.

(**A & B**) Morphine consumption and physical signs measured in female breeders while having access to a single bottle containing morphine (forced morphine exposure). (**A**) Forced morphine intake during the initial phase of treatment, when morphine dams' solution is ramped up from 0.1 mg/mL morphine to 0.2 mg/mL morphine, respectively (n=11). (**B**) Total physical signs in morphine-exposed dams at baseline (pre-treatment), and 8 hours after withdrawal from forced morphine exposure (n=10). (**C-E**) Morphine intake and physical signs during the C2BC paradigm. (**C**) Morphine intake in the C2BC paradigm during weeks 2-8. (n=10-11/week) * $p<0.05$, **$p<0.01$ compared to Week 2; # $p<0.05$, ## $p<0.01$ compared to Week 3. (**D**) Total physical signs for morphine-exposed dams while morphine sated in the C2BC paradigm and 8 hours after withdrawal from morphine (n=10). (E) Comparison of total physical signs in control, saccharin-drinking dams and morphine-drinking dams 8 hours after morphine withdrawal in the C2BC paradigm (n=12,10).

** $p<0.01$, ***$p<0.001$, **** $p<0.0001$; Mor= Morphine, WD= Withdrawal, C2BC= Continuous Two-Bottle Choice, Sacc= Saccharin

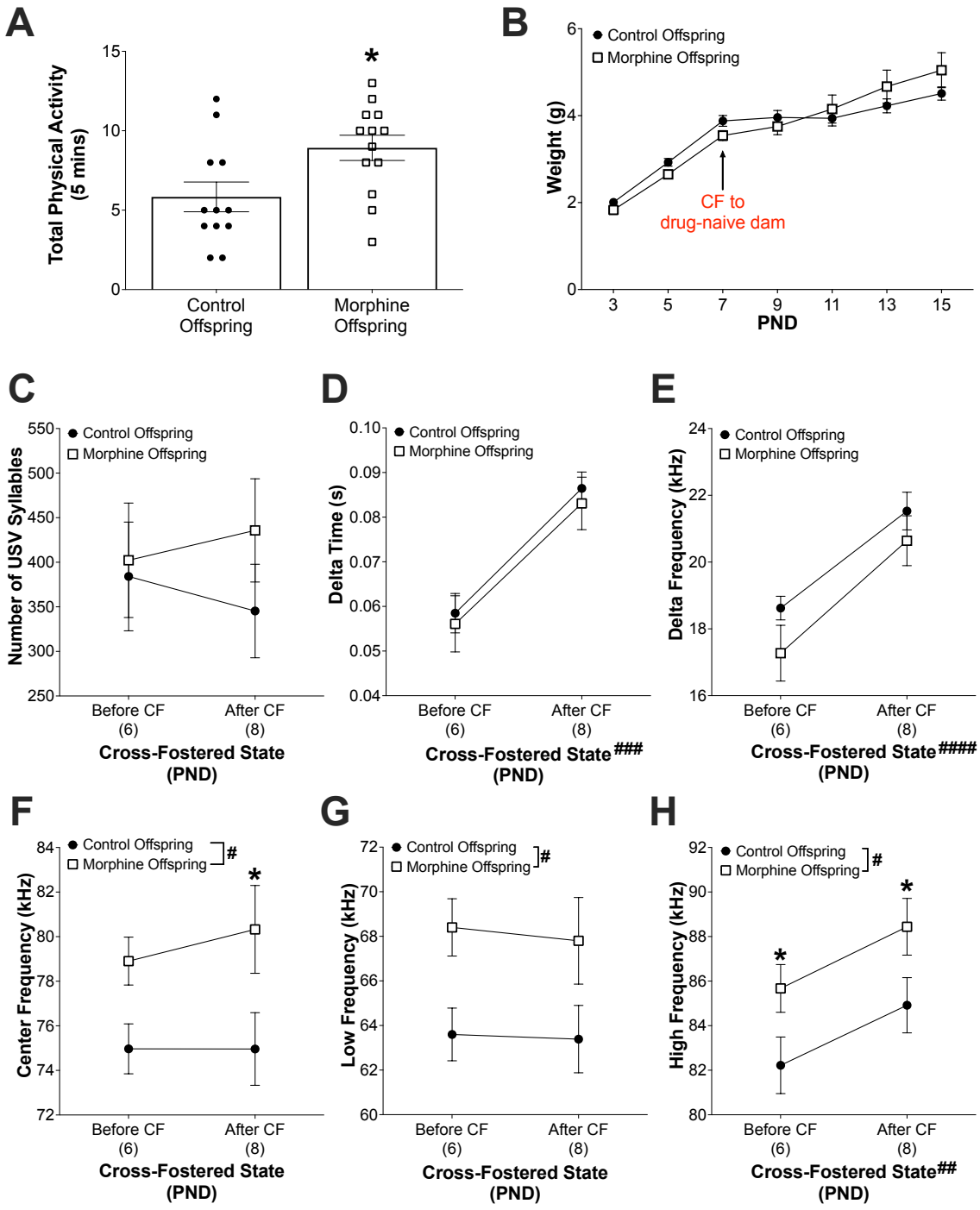


Figure 2-3: Behavioral outcomes in PND 2-15 pups before and after cross-fostering.

(**A**) PND 3 morphine offspring exhibited greater physical signs than control offspring (n=12,13). (**B**) Offspring body weight before and after cross-fostering (PND 3-15); (10-18 litters/PND). (**C**) Average number of USV syllables before and after CF (litter n= 9,7). (**D**) Average delta time (s), or time duration, of each USV call (n= 9,7). **(E)** Average delta frequency (Hz), or frequency range of each USV call (litter n= 9,7). (**F**) Average center frequency (Hz), or middle frequency for each USV call (litter n= 9,7). (**G**) Average low frequency (Hz) for each USV call (litter n= 9,7). (**H**) Average high frequency (Hz) for each USV call (litter n= 9,7).

PND= Postnatal day, CF= Cross-Fostered

# indicates main effect: # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001; * indicates post-hoc significance: * p<0.05

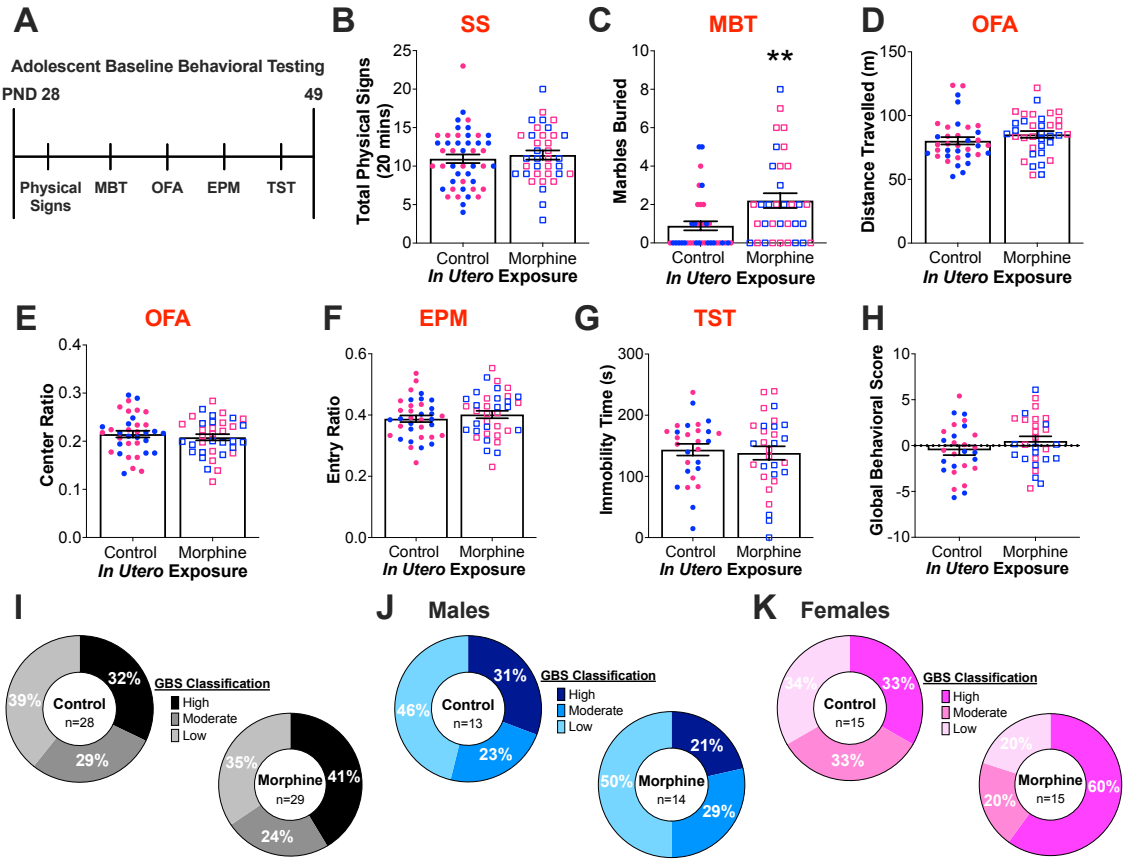


Figure 2-4: Sex-specific changes in baseline behavior in adolescent offspring from morphine-exposed dams.

(A) Experimental scheme showing the sequence of behavioral tests for the analysis of PND 28-49 adolescent offspring. (**B**) Average number of total physical signs in control and morphine-exposed offspring (n=46, 36). (**C**) Average number of marbles buried in the Marble Burying Test (MBT) (n=37, 34). (**D**) Average distance travelled (m) in the Open Field Arena (OFA) (n=36, 36). **(E)** Average center distance ratio in the OFA (n=36, 36). (**F**) Average open arm entry ratio in the Elevated Plus Maze (EPM) (n=36, 37). (**G**) Average immobility time (s) in the Tail Suspension Test (TST) (n=28, 30). (**H**) Average global behavioral score (GBS) calculated as the summation of all the *z*-scores for each behavioral test for each animal (n=28, 29). Percent of offspring from control and morphine-exposed dams (**I**), and male (**J**) and female **(K**) offspring that classified as high, moderate, and low scorers based on their global behavioral score for baseline behavior. **p<0.01

PND = Postnatal Day; SS = Somatic Signs; Blue symbols = males; Pink symbols = females

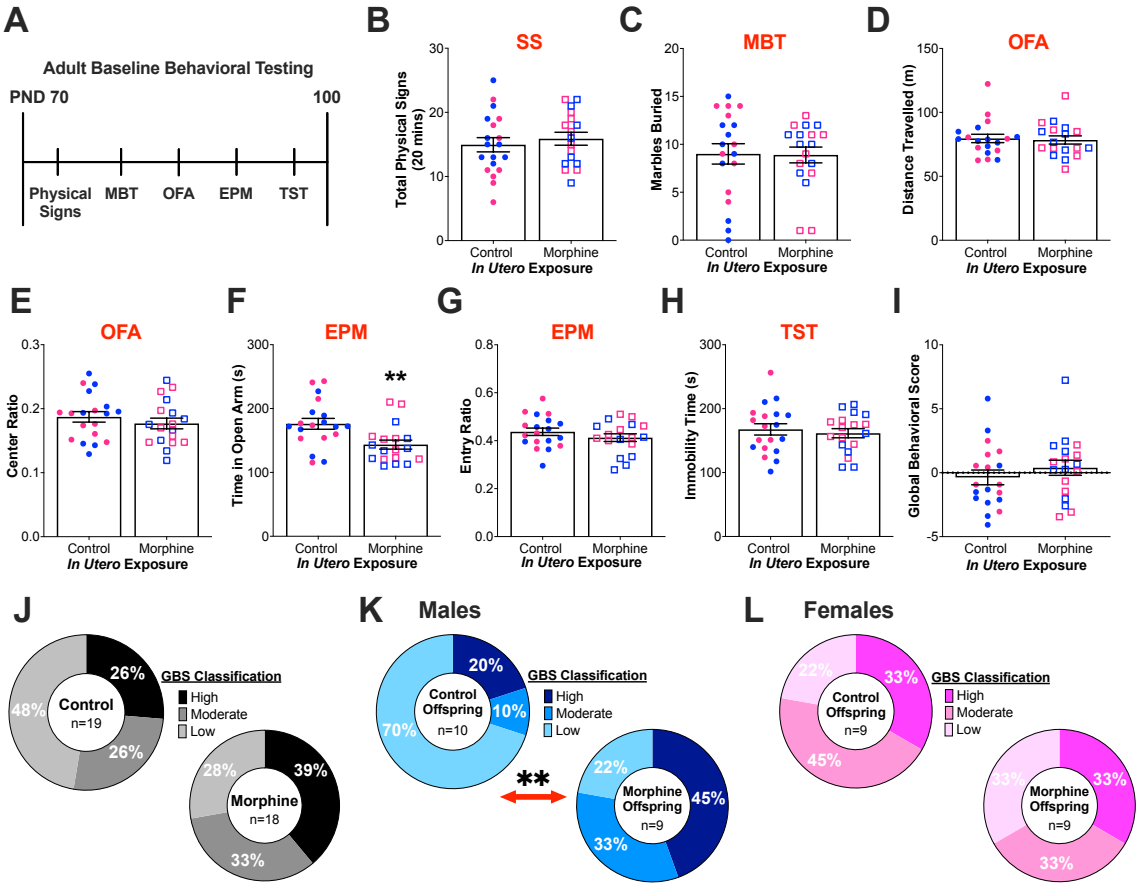


Figure 2-5: Sex-specific changes in baseline behavior of adult offspring from morphine-taking dams.

(**A**) Experimental scheme for the analysis of adult offspring in a battery of behavioral tests. (**B**) Average baseline total physical signs observed in offspring (n=19, 17). (**C**) Average marbles buried in the Marble Burying Test (MBT) (n=19, 18). (**D**) Average distance travelled (m) in the Open Field Arena (OFA) (n=19, 18). **(E)** Average center distance ratio in the OFA (n=19, 18). (**F**) Average time in the open arms of the Elevated Plus Maze (EPM) (n=19, 18). (**G**) Average open arm entry ratio in the EPM (n=19, 18). (**H**) Average immobility time (s) in the Tail Suspension Test (TST) (n=19, 18). (**I**) Average global behavioral scores (GBS) calculated as the summation of all the z-scores for each behavioral test for each animal (n=19, 18). Percent of control and morphine-exposed offspring (**J**), male (**K)**, and female (**L**) offspring that classified as high, moderate, and low scorers based on their GBS for baseline behavior.

**p<0.01; SS = Somatic Signs; Blue symbols = males; Pink symbols = females

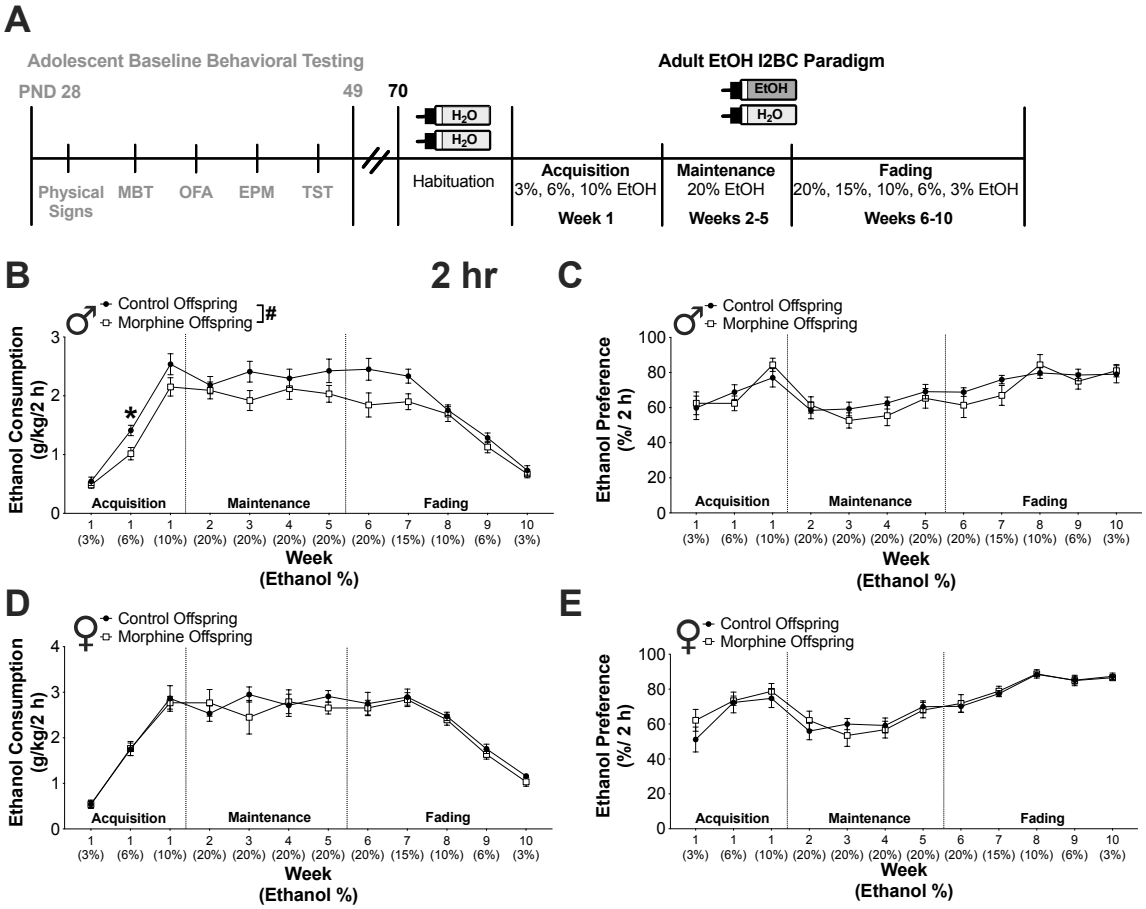


Figure 2-6: Ethanol 2-hour intake and preference for male and female offspring in the I2BC paradigm.

(**A**) Schematic of the adult ethanol I2BC experimental timeline after mice underwent baseline adolescent behavioral testing. (**B & C**) Average 2-hr ethanol intake (g/kg) **(B)** and preference (%) (C) for male offspring during weeks 1-10 of ethanol drinking (n=14). **(D & E)** Average 2-hr ethanol intake (g/kg) **(D)** and preference (%) **(E)** for female offspring during weeks 1-10 of ethanol drinking (n=12).

# p<0.05 main effect of dam treatment for 'Acquisition' and 'Fading' phase; * indicates post-hoc significance: * p<0.05

PND = Postnatal Day; MBT = Marble Burying Test; OFA = Open Field Arena; EPM = Elevated Plus Maze; TST = Tail Suspension Test; I2BC = Intermittent Two-Bottle Choice

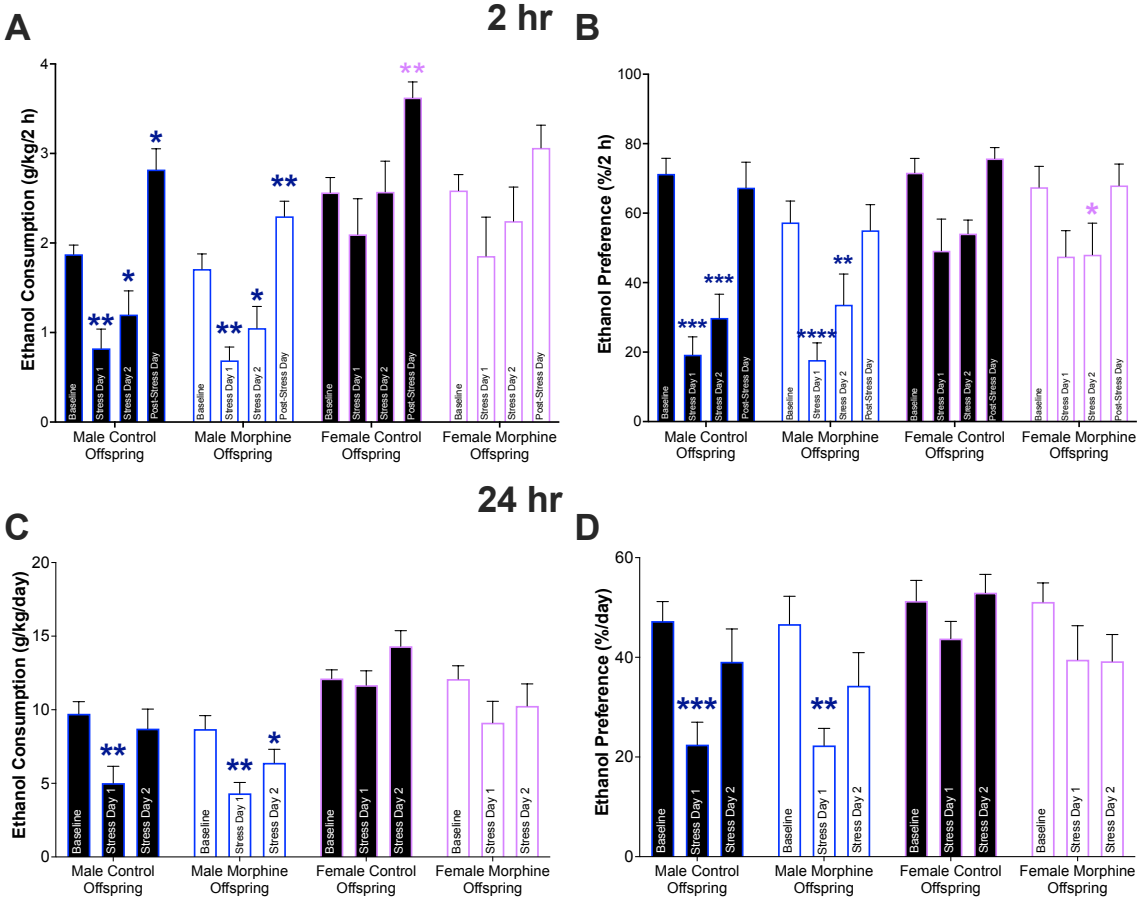


Figure 2-7: The effect of two days of restraint stress on ethanol 2-hour and 24-hour intake and preference for male and female offspring in the I2BC paradigm.

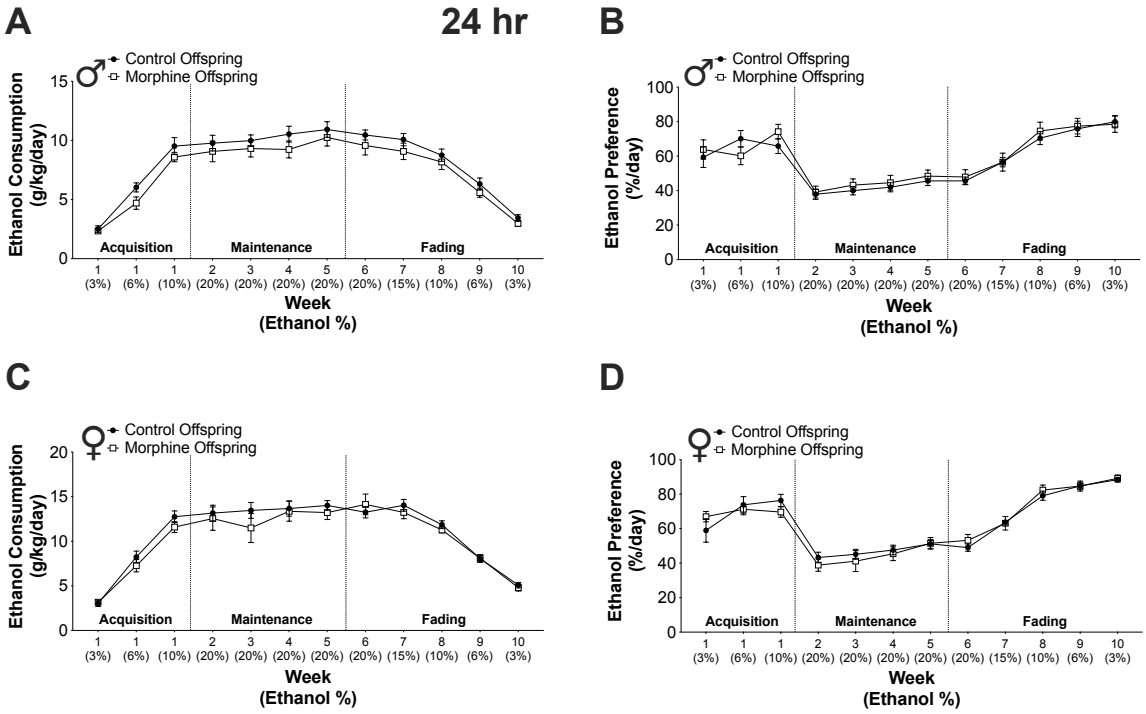
(A & B) Average 2-hr ethanol intake (g/kg) (**A**) and preference (%) **(B)** for male (n=10) and female (n=8,9) offspring during a 20% ethanol pre-stress baseline (week 11), after two-days of a 1-hour restraint stress (stress day 1 and 2), and on a day no stress was

given (post-stress day). **(C & D)** Average 24-hr ethanol intake (g/kg) (**C**) and preference (%) **(D)** for male (n=10) and female (n=8,9) offspring during a 20% ethanol pre-stress baseline (week 11), and after two-days of a 1-hour restraint stress (stress day 1 and 2).

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, compared to offspring's Baseline

I2BC = Intermittent Two-Bottle Choice

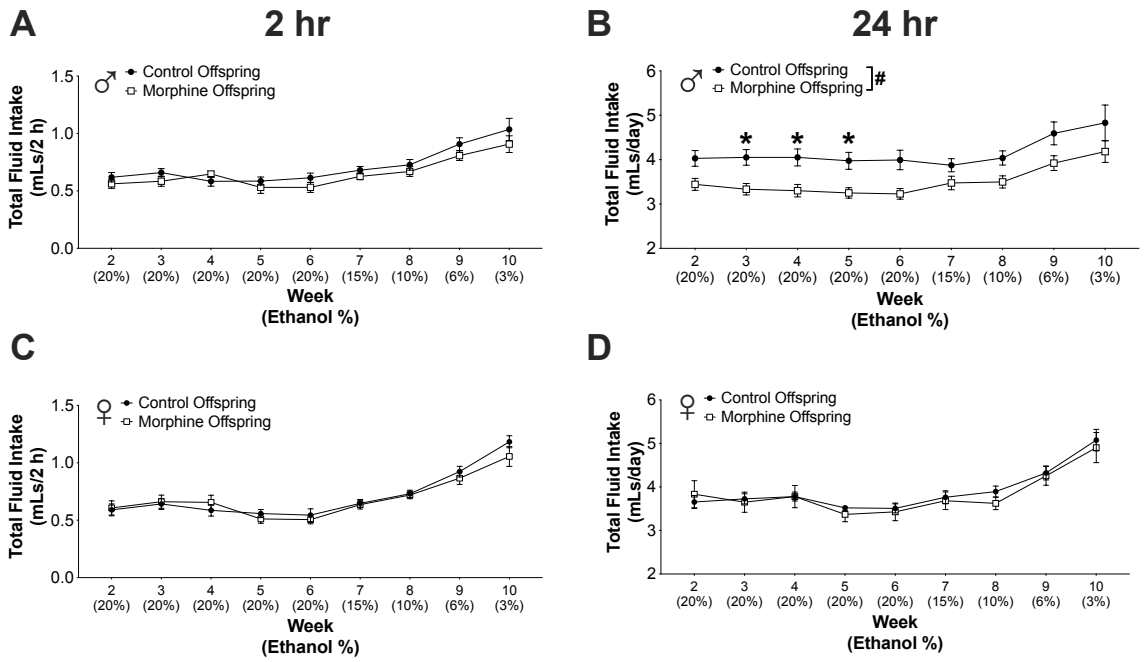
2.7. Supplemental Figures



Supplemental Figure 2-1: Ethanol intake and preference (24-hour) for male and female offspring in the I2BC paradigm.

(A & B) Average 24-h ethanol intake (g/kg) **(A)** and average 24-h ethanol preference (%) **(B)** for male offspring during weeks 1-10 of drinking (n=14). (**C & D)** Average 24-h ethanol intake (g/kg) **(C)** and ethanol preference (%) **(D)** for female offspring during weeks 1-10 of drinking (n=12).

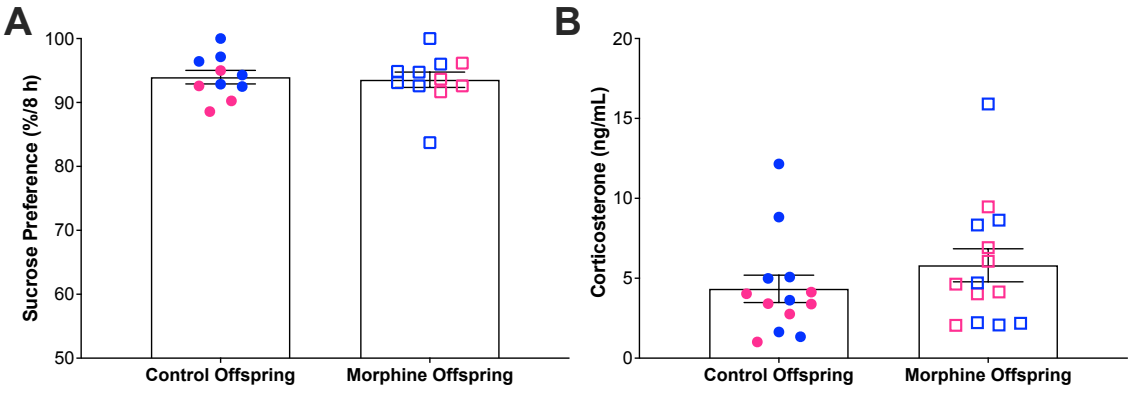
I2BC = Intermittent Two-Bottle Choice



Supplemental Figure 2-2: Total fluid intake (2- and 24- hour) for offspring in the I2BC paradigm.

(A) Average 2-hour total fluid intake (mL) for male offspring from weeks 2-10 (n=14). (**B**) Average 24-hour total intake (ml) for male offspring from weeks 2-10 (n=14). (**C**) Average 2-hour total fluid intake for female offspring from weeks 2-10 (n=12). (**D**) Average 24-hour total fluid intake for female offspring from weeks 2-10 (n=12).

# p<0.05 main effect of dam treatment; * indicates post-hoc significance: * p<0.05



Supplemental Figure 2-3: Investigating if offspring have changes in anhedonia-like behavior and dysregulated HPA axis functionality after the ethanol I2BC paradigm.

(A) Average sucrose preference, measured by the SPT after offspring underwent the ethanol I2BC paradigm (n=10,11). (**B**) Average corticosterone levels in offspring that were ethanol sated during the ethanol I2BC and underwent the DST to test the functionality of the HPA axis (n=13,14).

SPT = Sucrose Preference Test; DST = Dexamethasone Suppression-Test

Table 2-1: Behavioral correlations for morphine dams and their offspring.

Subject	Comparison 1	Comparison 2	r
Morphine dam	Mor intake (mg/kg) during 1st week of C2BC paradigm	Total SS during spontaneous Mor WD after 1 week of C2BC paradigm	-0.56 **
	Mor intake (mg/kg) during 1st week of C2BC paradigm	Mor intake (mg/kg) during 0.2 mg/mL forced Mor phase	0.49 **
	Mor intake (mg/kg) during 0.2 mg/mL forced Mor phase	Total SS during spontaneous Mor WD after forced Mor phase	-0.69 ****
Adolescent offspring from morphine-dams	Offspring number of marbles buried	Dam mor intake (mg/kg) during 1st week of C2BC paradigm	-0.54 ***
	Offspring number of marbles buried	Dam mor intake (mg/kg) during 0.2 mg/mL forced Mor phase	-0.37 *
	Offspring number of marbles buried	Dam total SS during spontaneous Mor WD after 1 week of C2BC paradigm	0.51 **
	Offspring OFA total distance travelled	Dam total SS during spontaneous Mor WD after forced Mor phase	-0.36 *
	Offspring OFA center distance ratio	Dam mor intake (mg/kg) during 1st week of C2BC paradigm	0.35 *
	Offspring EPM entry ratio	Dam mor intake (mg/kg) during 1st week of C2BC paradigm	0.39 *
	Offspring TST immobility time	Dam total SS during spontaneous Mor WD after forced Mor phase	-0.54 **

Pearson's correlation test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Abbreviations: Mor = Morphine, C2BC = Continuous Two-Bottle Choice, SS = Somatic Signs, OFA = Open Field Arena, EPM = Elevated Plus Maze, TST = Tail Suspension Test

CHAPTER 3: Prenatal-Preweaning Morphine Exposure Alters Baseline Behavior, Prefrontal Cortex Gene Expression, and Ethanol Preference in Female Mice

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This manuscript is currently in preparation.

3.1. Introduction

With the rise in Opioid Use Disorder (OUD) around the world, the rate of pregnant women that use opioids have also increased (Jarlenski et al., 2021). A proportion of newborns exposed to *in utero* opioids experience Neonatal Opioid Withdrawal Syndrome (NOWS) and require pharmacological interventions to reduce NOWS symptoms, which include tremors and body weight changes (Bailey et al., 2022; Weller et al., 2020; Zimmermann et al., 2020). The fact that a subset of newborns develops NOWS after *in utero* exposure and/or has a higher prevalence for early-life complex chronic conditions (Jarlenski et al., 2020) raises the concern that there are vulnerable sub-populations among these children that could be more susceptible to long-term adverse outcomes.

Reviews and meta-analysis have reported that some children exposed to *in utero* opioids display changes in cognition, hyperactivity, and impulsivity in early childhood, although outcomes in adulthood are less understood (Weller et al., 2020). Preclinical studies have attempted to shed light on the long-term effects of maternal morphine exposure on offspring's behavior, including developmental and physical milestones (Byrnes and Vassoler, 2018). The maternal paradigms used in those preclinical studies vary in opioid dose used, drug administration length, and drug delivery method, which has led to the report of varied effects in offspring behavior. Therefore, the long-term consequences of maternal opioid exposure on adolescent and adult behavior, including drug misuse, remain unclear.

Considering that both morphine exposure and withdrawal can change brain transcriptomics in humans and animal models (Borrelli et al., 2021; Liu et al., 2021;

Mayberry et al., 2022; Seney et al., 2021), we were interested in assessing brain-region specific transcriptomic changes associated with behavioral changes and phenotypes observed in morphine-exposed offspring. Previous studies investigating transcriptomic alterations in offspring after maternal opioid use have shown changes in hepatic tissue (Toorie et al., 2022), saliva (Yen et al., 2019), and placenta (Radhakrishna et al., 2021), and *in utero* exposure to opioids has been shown to affect fetal brain miRNAs in a sex-dependent fashion (Goetzl et al., 2019). In addition, pups injected with morphine from postnatal day (PND) 1-14 were reported to have transcriptomic changes in the brainstem (Borrelli et al., 2021). However, no studies have evaluated transcriptomic changes in offspring's prefrontal cortex (PFC) after prenatal-preweaning morphine exposure. The PFC is a critical brain region altered by endogenous and exogenous opioids which, in combination with other brain regions, is important for cognition and drug-related associative memories (Abraham et al., 2021; Li et al., 2018; Rosen et al., 2015). In addition, transcriptional signatures in the PFC have been shown to distinguish vulnerable and resilient populations after drug exposure (Navandar et al., 2021). And, one clinical study showed alterations in thalamus to frontal cortex connectivity in newborns exposed to prenatal opioids, which also correlated with NOWS severity (Radhakrishnan et al., 2022).

Despite widely known alcohol-opioid interactions (Gianoulakis, 2001; Job et al., 2007; Oslin et al., 2006), the effect of maternal morphine exposure on offspring's alcohol use risk is largely unknown. One study reported that a significantly higher proportion of individuals whose mothers used heroin during pregnancy reported misusing alcohol during their lives (Nygaard et al., 2020). In addition, only one preclinical study to-date has reported that offspring exposed prenatally to methadone have altered sensitivity to

alcohol reward and alcohol binge-drinking, measured using conditioned place preference and a drinking-in-the-dark paradigm (Grecco et al., 2022). So far, no preclinical study has investigated the consequences of prenatal-preweaning morphine exposure on alcohol's reinforcing effects using an oral ethanol intermittent two-bottle choice (I2BC) paradigm.

Using a model of maternal opioid use, our findings demonstrate sex-specific alterations in adolescent and adult behavior, including ethanol preference in the I2BC paradigm and transcriptomic changes in the PFC.

3.2. Materials & Methods

3.2.1. Animals

C57BL/6J mice were group-housed in standard plastic cages with filter cage tops and corn-cob bedding. They were given *ad libitum* food (Labdiet, 5053, PMI, Brentwood, MO) and water. Animals were housed in a temperature and humidity-controlled 12-hour reverse-light cycle room (lights "off" at 10:00 AM; 65-75 ^{o}F, 40-60% relative humidity). Mice evaluated for drug use risk (dams and offspring subsets) were single housed with Shepherd Shacks® (Shepherd Specialty Papers, Richland, MI) as enrichment at the start of their drinking experiment to ensure accurate measure of drug dose. For all drinking experiments, bottle placement was alternated daily to limit side preference. Bottles were weighed daily to account for fluid leakage due to handling of cages. Solutions for drinking experiments were presented in 50-mL conical tubes with rubber stoppers and stainless-steel sippers. Animals were tested during the dark phase of the light cycle and habituated to the testing room at least 30 minutes before the start of the

test. Luminosity, reported as lux, of the testing room was measured using a digital light meter (LX-105 Lutron Digital Light Meter). All experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

3.2.2. Prenatal-Preweaning Morphine Treatment

Female mice were single-housed and habituated to one bottle of 0.2% saccharin (Saccharin Sodium Salt Hydrate, Sigma, St. Louis, MO) in filtered water. After this first week of habituation, mice were evaluated for 30 minutes for baseline physical signs. Signs evaluated included those commonly reported for rodent morphine withdrawal: wet dog shakes, jumping, head shakes, teeth chattering, writhing, and diarrhea (Muldoon et al., 2014). Mice were then assigned to a control-saccharin group ("control dam") and a morphine-treatment group ("morphine dam"), ensuring that both groups of mice had on average similar baseline physical signs to start. Mice assigned to the morphine-treatment group were then given one bottle of 0.1 mg/mL free-base (f.b.) morphine (Morphine Sulfate, Spectrum Chemical MFG. Corp, New Brunswick, NJ) in 0.2% saccharin water for four days. The concentration was then escalated to 0.2 mg/mL f.b. morphine for 3 days. This forced exposure phase ensured female mice would become dependent on morphine. Control-saccharin female mice were maintained on one bottle of 0.2% saccharin during the 'forced' exposure phase. To confirm dependency on the last day of the forced morphine (0.2 mg/mL) phase, the morphine bottle was replaced with a 0.2% saccharin bottle and 8 hours later, mice were evaluated for spontaneous physical signs of morphine withdrawal (detailed above). Control-saccharin females were also evaluated for physical signs at this time.

Female mice were then transitioned to a continuous two-bottle choice paradigm (C2BC) where they had access to one bottle of filtered water and one bottle of morphine + saccharin (0.1 mg/mL f.b. morphine in 0.2% saccharin). Control-saccharin females received one bottle of water and one bottle of saccharin (0.2%) fluid. After one week in the C2BC, mice were evaluated for spontaneous physical signs of withdrawal 8 hours after the removal of the morphine bottle, and while morphine sated two days later. Control-saccharin females were also evaluated for physical signs.

To solely study the effects of maternal opioid exposure on offspring behavior, both control-saccharin and morphine-treated female mice were paired with single-housed male C57BL/6J mice (~5 hours/day), and then returned to their own cage to continue their C2BC paradigm. Female breeders were paired with male breeders until pregnancy was confirmed by substantial body weight gain. Female breeders were maintained on the C2BC paradigm throughout gestation and from offspring PND 0 - 21. To study the effects of opioid use on what would be comparable to the full-term pregnancy in humans, we use a maternal morphine oral self-administration paradigm that begins pre-pregnancy and continues throughout gestation and until offspring postnatal day (PND) 21, since mouse development continues postnatally (Craig et al., 2003; Semple et al., 2013). This approach was based on the fact that brain maturation and developmental milestones, such as synapse formation and myelination, which occur during gestation for a human fetus, occur during early PNDs for mice (Richard and Flamant, 2018; Ross et al., 2015; Semple et al., 2013). Daily morphine consumed by female breeders was reported as morphine intake (mg)/mouse body weight (kg), and preference (%) was calculated as (mL morphine consumed/mL total fluid consumed)*100.

Offspring body weight was recorded on PND 3, 5, 7, 11, 13, and 15. To limit older offspring (PND 17-21) from drinking from their dam's treatment bottle, treatment bottles were placed in metal holders at a 90° angle so that only dams could reach them, while the water bottle was at an angle that offspring could reach. Offspring from control dams and morphine dams were weaned and group-housed at PND 21. An average of 8-9 litters per dam treatment were used in the studies presented.

3.2.3. Assessment of Adolescent Baseline Behavior

Offspring were acclimated to handling at least five days before adolescent testing began. Testing occurred between PND 28 - 49. To assess baseline physical signs, offspring were placed in a novel cage with clean bedding and a filtered cage top (room lux: 2). As previously described (Perez and De Biasi, 2015), adolescent offspring were observed undisturbed for 20 minutes for the following baseline physical signs: shaking, scratching, grooming, and teeth chattering. These cages were then used for the marble burying test (MBT), with 5 centimeters of bedding and 20 marbles evenly spaced on top. Offspring were left undisturbed for 30 minutes (room lux: 2) and the number of marbles buried (fully buried or at least 2/3 buried) were recorded to assess anxiety-like/compulsive-like behavior as described before (Perez and De Biasi, 2015).

Offspring were tested in the Open Field Arena (OFA) at least 48 hours after the MBT. The OFA apparatus was made of white opaque plexiglass (40 cm x 40 cm), which was divided into a center zone (20 cm x 20 cm, lux: 4) and a surround zone (10 cm all around walls, lux: 2) (Perez and De Biasi, 2015). Offspring were placed at the center of the OFA and allowed to freely explore for 30 minutes while being recorded with an

ANYMAZE behavioral tracking system (Stoeling Co, Wood Dale, IL). Locomotion was assessed using the average total distance travelled (m). Anxiety-like behavior was reported as time spent in the center zone (s), and center distance ratio [distance travelled in center zone (m)/total distance travelled (m)].

The day after the OFA test, mice were placed in the same OFA apparatus for the training phase of the Novel Object Recognition Test (NORT). During training, mice were tracked for 10 minutes as they explored two similar objects (two circle or two rectangle objects). To assess changes in long-term memory, mice were placed back in the OFA 24 hours after their training phase, where time exploring a novel object (circle or rectangle object) was recorded for 5 minutes. Average discrimination index is calculated as [time spent exploring novel object (s) – time exploring training object (s)]/total exploration time (s). An experimenter blinded to offspring dam treatment manually scored training and testing phase videos.

At least 48 hours after NORT, offspring were tested in the Elevated Plus Maze (EPM) for 10 minutes (open arms: 4 lux, closed arms: 1 lux) as previously described (Perez and De Biasi, 2015). For an entry into an arm to be recorded by ANYMAZE, 70% of the mouse's body must be inside the arm. Anxiety-like behavior was assessed by the average time spent in the open arms (s) and open arm entry ratio [open arm entries/(open arm entries + closed arm entries)]. Offspring were tested in the Tail Suspension Test (TST) for six minutes (room lux: 4) to measure depressive-like behavior, at least 48 hours after the EPM (Gangitano et al., 2009). Average time spent immobile (s) was reported.

3.2.4. Assessment of Adult Baseline Behavior

A separate cohort of adult (~2 months old) group-housed offspring from morphine-treated and control dams were tested in the battery of behavioral tests described above. Offspring were observed for 30 minutes for baseline physical signs, which included: jumping, shaking, head nods, scratching, grooming, and teeth chattering. Offspring were also assessed in the MBT, OFA, NORT, EPM, and TST.

3.2.5. Global Behavioral Score Classification

To assess behavior holistically, we utilized seven baseline behavioral measures to calculate adolescent and adult offspring global behavioral scores (GBS). These include: (1) physical signs, calculated as the total number of physical signs, (2) anxiety-like/compulsive-like behavior, calculated as total number of marbles buried in MBT, (3) locomotion, calculated as total distance travelled in OFA, (4) anxiety-like behavior in OFA, calculated as center distance ratio, (5) anxiety-like behavior in EPM, calculated as open arm entry ratio, (6) depressive-like behavior in the TST, calculated as total immobility time, and (7) cognitive deficits in the NORT, reported as discrimination index. As previously described in Quijano Cardé and De Biasi (2022), the raw behavioral scores were standardized to z-scores. The z-score was then multiplied by the direction (+1 or -1) for that behavioral measure, to indicate a worst score. Z-scores for each offspring were summed to obtain a GBS. GBS classifications for offspring were defined as: ‘high’ (GBS > +1), ‘moderate’ (-1 ≤ GBS ≤ +1), ‘low’ (GBS < -1). Only offspring with raw data for all behavioral measures were used for this analysis.

3.2.6. Assessment of Ethanol Use Risk using the Ethanol intermittent Two-Bottle Choice (I2BC) Paradigm

Offspring tested for baseline behaviors during adolescence were allowed to mature into adulthood (~2 months old) and single-housed for the duration of the I2BC ethanol drinking experiment. As described previously (Hwa et al., 2011; Quijano Cardé and De Biasi, 2022), mice were habituated to two bottles of filtered water and to being single-housed for one week. Mice were then given one week of escalating concentrations of ethanol (v/v) on Monday (3% ethanol), Wednesday (6% ethanol), and Friday (10% ethanol), which was termed the 'Acquisition' phase. Offspring were then given access to 20% ethanol during weeks 2-5, which was termed the 'Maintenance' phase. One bottle of filtered water and one bottle of ethanol in water were always available in an intermittent schedule throughout the paradigm. Ethanol solutions were presented 3 hours into the dark phase of their light cycle, and 2- and 24- hour ethanol intake (g ethanol consumed/kg mouse body weight), and ethanol preference (%) [(ml ethanol intake/ml total fluid intake)*100] were calculated. Mice were weighed once a week to calculate ethanol dose consumed.

3.2.7. Tissue Collection

Brain tissue was collected from a subset of control and morphine-exposed mice (~PND 55) after adolescent baseline behavior was recorded. Prefrontal Cortex (1.0 mm punch) was collected after live decapitation and immediately frozen and stored at -80°F. Tissue was subsequently processed for bulk RNA-sequencing.

3.2.8. Bulk RNA-Sequencing

Total RNA was purified from adult mouse dentate gyrus using Rneasy kit (Qiagen), and Dnase I on-column digestion was performed to avoid genomic DNA contamination. Sequencing libraries were prepared using NEBNext Ultra RNA Library Prep kit for Illumina (E7530L) following manufacturer's protocol. Briefly, total RNA was poly-A tail selected and then heat fragmented. The fragmented RNA was reverse transcribed and the second strand was synthesized to make double stranded DNA. After end repair and 3' adenylation, adapters for multiplexing were ligated to the end of double stranded DNA fragments. The ligation products were amplified and purified to generate illumina compatible libraries. Sequencing was performed with 75bp-single end sequencing by illumina NextSeq 550. The raw reads were mapped to the mouse genome build mm10 using STAR (Dobin et al., 2013). The differential gene expression and downstream analyses were performed using DESeq2 (Love et al., 2014) and custom R scripts.

3.2.9. Statistical Analyses

Data are expressed as mean +/- standard error of the mean and were analyzed using Graphpad PRISM 9. A p-value of <0.05 was considered statistically significant. ROUT (Q=1%) in Prism was used to remove significant outliers. Litter averages are shown for neonatal body weight data, while individual points are shown for dam, adolescent, and adult data when appropriate. A Two-Way ANOVA was used to assess a main effect and/or interaction of dam treatment and sex on offspring behavior. A repeated-measures (RM) three-way ANOVA for ethanol I2BC drinking revealed a main effect of sex, so females and male drinking data were analyzed separately with a RM

two-way ANOVA. For the ethanol I2BC experiment, the 'Acquisition' and 'Maintenance' phase were analyzed separately. A mixed-effects model was used for any data set that was missing values at a specific timepoint (ex. bottle pushed out, litter could not be weighed, etc.). The percent of offspring that classified under each GBS classification ('high', 'moderate', 'low') was assessed using the outcome vs. expected chi-square test, where the control offspring percentage was considered 'expected' prevalence. Where appropriate, a Sidak test was used for post hoc analysis.

3.3. Results

3.3.1. No change in offspring bodyweight after prenatal-preweaning morphine exposure

Utilizing a preclinical paradigm to model OUD in humans, similar to methods used in a preprint by Fleites *et al.* (2022), female mice were given morphine pre-pregnancy to establish dependence. Female dams significantly escalated their morphine intake when the morphine concentration was ramped up (0.1 mg/mL-0.2 mg/mL) during the forced morphine exposure in week 1 of the paradigm (paired t-test; t = 7.962, df = 13, p = <0.0001; Figure 3-1A). Mice were then transitioned to a continuous two-bottle choice (C2BC) morphine paradigm throughout gestation and until offspring PND 21, so that offspring received morphine while *in utero* and postnatally, through lactation. There was a significant main effect of week (RM one-way ANOVA; F(1.963, 25.52)= 7.321, p = 0.0032; Figure 3-1B) for dams' morphine intake, where it seems that the decrease in morphine intake during weeks 3-6 could be due to the mating procedure (weeks 3-4) and to increased body weight while dams were pregnant (weeks 5-7). In addition, we assessed offspring body weight while they lactated from their dams and observed that

offspring from morphine-treated dams had similar body weights to control offspring during PND 3-15, where body weight increased across PNDs for all offspring (Figure 3-1C).

3.3.2. Subtle sex-specific changes in adolescent behavior

Offspring were tested during adolescence in a battery of tests to investigate changes in baseline behaviors (Figure 3-2A). Although there was no significant effect of dam treatment on number of physical signs, there was a main effect of sex (Two-way ANOVA; $F(1,94) = 6.517$; $p = 0.0123$; Figure 3-2B), where post-hoc analysis revealed that female offspring from morphine-treated dams display increased baseline physical signs compared to male offspring from morphine-treated dams. When anxiety-like/compulsive-like behavior was assessed using the MBT, we found no significant effect of dam treatment and a trend for a main effect of sex ($p=0.0998$; Figure 3-2C). When we assessed changes in locomotion using the OFA, we found a significant interaction between dam treatment and sex (Two-way ANOVA; $F(1,91) = 5.887$; $p = 0.0172$; Figure 3-2D), but no significant main effect of dam treatment or sex. Post-hoc analysis revealed that male offspring from morphine-treated dams displayed a trend ($p=0.0511$) for increased locomotion compared to male offspring from control dams. Offspring displayed no significant changes in baseline anxiety-like behavior in the OFA (Figure 3-2E), and a trend for an interaction between dam treatment and sex ($p=0.0655$; Figure 3-2G) in the EPM.

Next, we assessed learning and memory in our offspring using the NORT. During the training phase of the NORT, there was a main effect of sex (Two-way ANOVA;

F(1,67) = 6.328; p = 0.0143; Supplemental Figure 3-1A) for total time exploring the training objects, where post-hoc analysis revealed that female offspring from morphine-treated dams display increased total exploration time compared to male offspring from morphine-treated dams. Offspring were then assessed for deficits in long-term memory during the testing phase of the NORT. We found that offspring from morphine-treated dams did not display memory deficits compared to control offspring when we analyzed their object discrimination index (Figure 3-2F). Similarly, offspring displayed no significant changes in depressive-like behavior in the TST (Figure 3-2H).

Although there were no significant effects of dam treatment in the individual behaviors examined, we were interested in characterizing overall offspring's behavioral phenotype by computing a composite score that encompasses multiple behaviors (El-Kordi et al., 2013; Fleites et al., 2022; O'Neal et al., 2020; Quijano Cardé and De Biasi, 2022). As shown in Figure 3-2I, overall, offspring from morphine-treated dams displayed no significant difference in GBS compared to offspring from control dams. When we explored the distribution of offspring GBS, characterized into 'high', 'moderate', and 'low' phenotypic scores, we found that a similar percent of male offspring from control dams and morphine-treated dams fell into the 'high' GBS classification (~21-29%; Figure 3-2J). Interestingly, there was a trend (p=0.0531) for a higher percent (50%) of female offspring from morphine-treated dams to classify under the 'high' GBS classification, compared to 26% of females from control dams (Figure 3-2K). This suggests that female offspring from morphine-treated dams display a slightly worst behavioral severity phenotype during adolescence when their behavior is observed holistically.

3.3.3. Gene expression analysis in the Prefrontal Cortex of offspring from morphine-treated dams during late adolescence

To determine whether the subtle changes in adolescent behavior were associated with transcriptomic changes in the brain, brain tissue was collected and processed for bulk RNA-sequencing from a subset of mice (~PND 56) that underwent adolescent behavioral testing. Due to the shift to a more severe GBS phenotype in female offspring from morphine-treated dams, we were particularly interested in investigating potential changes in the Prefrontal Cortex (PFC), based on the fact that activity in the PFC has also been shown to predict individual vulnerability/resiliency to stress (Kumar et al., 2014) and substance misuse (Ersche et al., 2020). In addition, the PFC is important for executive function and processing reward value, and its connectivity and functionality is altered after exposure to external factors, including drugs and stress (Abraham et al., 2021; Lutz and Kieffer, 2013; Rosen et al., 2015).

Bulk RNA-sequencing in the PFC revealed various gene transcripts that were significantly upregulated and downregulated between same-sex offspring from morphine-treated dams and control offspring (Figure 3-3A; Supplemental Figure 3-4; Supplemental Table 3-1 and 3-2). Specifically, 134 genes were upregulated in female offspring, while 43 genes were downregulated in male offspring (Figure 3-3B). While 14 genes were downregulated in female offspring and 13 genes were downregulated in male offspring (Figure 3-3B). In addition, among the differentially expressed genes (DEGs) that were upregulated, carbonic anhydrase 13 (Car13) had one of the highest fold changes (4.4-fold) in female offspring from morphine-treated dams, compared to female control offspring (Figure 3-3A). Car13 is an enzyme that only recently started to be characterized (Lehtonen et al., 2004), and has the potential to be further investigated

in offspring from morphine-treated dams. In addition, we found genes enriched in extracellular matrix (ECM) receptor interaction and focal adhesion pathways to be specifically upregulated in female offspring with morphine exposure, such as Tnxb, Sv2c, Col1A2 and Col6a3 (Figure 3-3C). The heat-map analysis in Figure 3-3D also depicts the GBS for each offspring used for the transcriptomic analysis, showing the variety of GBS scores and its relationship to genes enriched in Figure 3-3C.

We investigated the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway terms for the gene transcripts that were either upregulated or downregulated. There were various significantly upregulated KEGG pathway terms in the PFC of female offspring from morphine-treated dams compared to female offspring from control dams (Table 3-1). Interestingly, the two pathways that had the highest fold change were the 'ECM receptor interaction' pathway (~16.45-fold change) and the 'protein digestion and absorption' pathway (~9.38-fold change). Only one KEGG Pathway term was significantly downregulated ($p=0.0054$) in male offspring from morphine-treated dams compared to control male offspring. The 'neuroactive ligand-receptor interaction' pathway had a 17.44-fold change with the following associated genes - S1pr4, Ntsr1, Hcrtr1.

Although our results point to DEGs that might underlie the shift to a more severe behavioral phenotype in female offspring from morphine-treated dams, further investigation will be needed to understand the role of those DEGS in the behavioral vulnerability of the offspring exposed to prenatal-preweaning morphine.

3.3.4. Decreased anxiety-like behavior in adult male offspring from morphine-treated dams

Adult offspring were evaluated for baseline somatic and affective changes in a battery of behavioral tests, as described in the previous section. There was no significant difference in baseline physical signs (Figure 3-4B), anxiety-like/compulsive-like behavior in the MBT (Figure 3-4C), locomotion and anxiety-like behavior in the OFA (Figure 3-4D-E), cognition (Figure 3-4F), and depressive-like behavior (Figure 3-4H). Surprisingly, in the EPM, there was a main effect of dam treatment (Two-way ANOVA; $F(1,34) = 6.480$; $p = 0.0156$; Figure 3-4G) and a trend for an interaction ($p = 0.0855$) between dam treatment and sex. Post-hoc analysis revealed that male offspring from morphine-treated dams had higher entry ratios in the EPM, suggesting decreased anxiety-like behavior. However, an alternative explanation for the decreased entry ratio could be that the mice exhibit more risk-taking or impulsive-like behavior, as has been reported in other studies (Koabel et al., 2021; Tucker and McCabe, 2021; Wille-Bille et al., 2017).

When we investigated offspring behavior using a standardized composite score, we found no changes between offspring in their GBS (Figure 3-4I). Next, we evaluated the percent of offspring that fell into each GBS classification and found a significant difference among male offspring (chi-square test; $df = 2$, $p = 0.0458$). A higher percentage (63%) of male offspring from morphine-treated dams fell into the ‘low’ GBS classification, while 30% of male control offspring had a ‘low’ behavioral severity phenotype (Figure 3-4J), although there were no differences among female offspring behavioral classifications (Figure 3-4K).

Together, this suggests that male offspring from morphine-treated dams have less anxiety-like behavior in the EPM, leading more of them to classify under the 'low' behavioral severity classification.

3.3.5. Female offspring from morphine-treated dams display decreased ethanol preference

Considering that ethanol is one of the most commonly misused substances and that there are known common mechanisms of action between ethanol and opioids (Gianoulakis, 2001; Job et al., 2007; Oslin et al., 2006), we decided to test whether offspring from morphine-treated dams would display changes in ethanol-related behaviors. Offspring that underwent adolescent behavioral testing were allowed to mature to adulthood and were then evaluated for ethanol misuse liability using the ethanol intermittent two-bottle choice (I2BC) paradigm.

Ethanol Intake (g/kg)

When male offspring's ethanol intake was evaluated in the 24-hour 'Acquisition' phase of the ethanol I2BC paradigm, we found a trend for an interaction between ethanol concentration and dam treatment ($p=0.0569$) (Figure 3-5A). We found no significant main effect of dam treatment for male offspring's 24-hour ethanol intake in the 'Acquisition' and 'Maintenance' phase (Figure 3-5A), and 2-hour ethanol intake during the 'Acquisition' and 'Maintenance' phase (Supplemental Figure 3-2A).

Female offspring's 24-hour ethanol intake during the 'Acquisition' and 'Maintenance' phase also revealed no significant effect or interaction of dam treatment (Figure 3-5C). Similar results were obtained for female offspring's 2-hour ethanol intake (Supplemental Figure 3-2C), where we found no significant main effect of dam treatment. In addition, there was no significant difference in 2-hour and 24-hour total fluid intake for male and female offspring (Supplemental Figure 3-3).

Ethanol Preference (%)

When male offspring's ethanol preference was evaluated in the ethanol I2BC paradigm, we found no significant main effect or interaction of dam treatment during the 24-hour 'Acquisition' and 'Maintenance' phase (Figure 3-5B). Similarly, there was no significant effect of dam treatment for male offspring's ethanol preference during the 2-hour 'Acquisition' and 'Maintenance' phase (Supplemental Figure 3-2B).

There was a significant effect of dam treatment for female 24-hour ethanol preference during the 'Acquisition' phase (RM Mixed-Effects Model; $F(1,21) = 7.350$; $p = 0.0131$; Figure 3-5D). Specifically, post-hoc analysis revealed that female offspring from morphine-treated dams have lower preference for 6% ethanol compared to female control offspring ($p = 0.0381$). Female ethanol preference during the 24-hour 'Maintenance' phase revealed a trend for a main effect of dam treatment ($p = 0.0689$) and a trend for an interaction of dam treatment and week ($p = 0.0559$) (Figure 3-5D). Female 2-hour ethanol preference for the 'Acquisition' phase revealed a trend for a main effect of dam treatment ($p = 0.0766$), and no difference during the 'Maintenance' phase (Supplemental Figure 3-2).

These results suggest that females, but not males, display lower 24-hour ethanol preference, despite no change in ethanol intake.

3.4. Discussion

Utilizing our voluntary oral morphine C2BC paradigm in dams, we demonstrated subtle sex-specific alterations in adolescent and adult offspring exposed to prenatal-preweaning morphine. Overall, the data presented support the hypothesis that maternal opioid exposure alters offspring behavior throughout development. Specifically, female offspring from morphine-treated dams display changes in their composite behavioral scores classification, their PFC transcriptome during late adolescence, and their ethanol preference during adulthood.

Our study did not detect changes in neonate body weight after prenatal-preweaning morphine exposure as we hypothesized, which is a common characteristic of newborns with NOWS (Weller et al., 2020). This could be due to our offspring not experiencing acute withdrawal during the PNDs when body weight was assessed and/or because our dams solely had exposure to one drug - morphine. A review by Isaacs et al. (2021) reported that most clinical studies where newborns had lower body weight and worst NOWS symptoms were in cases where mothers rather than using opioids alone during pregnancy, co-used opioids and nicotine (Isaacs et al., 2021). Poly-drug use during pregnancy is often observed among morphine users. For example, 80-90% of women on opioid maintenance therapy test positive for tobacco use (Goetzl et al., 2019). Therefore, the long-term developmental effects of morphine & nicotine co-abuse during pregnancy warrant further study (Zimmermann et al., 2020).

Clinical data suggests that a higher percentage of male offspring need pharmacotherapy to treat NOWS (Yen et al., 2019), which is why we hypothesized there would be changes in male offspring's behavior in our study. Instead, we observed subtle changes in female, but not male, behavior and PFC transcriptomics. This phenomenon might be influenced by the overall length of morphine exposure. In fact, using a maternal morphine paradigm that stops at offspring PND 7, when offspring are cross-fostered to a drug-naïve dam, we showed changes in male, but not female, offspring behavior (preprint, Fleites *et al*. 2022). Those results suggest that, when/if male offspring experience morphine withdrawal early in development (i.e. PND 7), when testosterone levels have been shown to peak in rodent brains (Clarkson and Herbison, 2016; Turano et al., 2019), the potential impact on their behavior is greater than that on females. In the present study where maternal opioid exposure continued until PND 21, male adult offspring from morphine-treated dams displayed decreased anxiety-like behavior in the EPM and a greater percentage of them fell in the lower GBS classification, further supporting the point of early withdrawal (i.e. PND 7) being important in seeing adverse outcomes in male offspring. It should also be noted that morphine has been shown to alter reproductive hormones, including estradiol, and gestational morphine leads to atrophy and hypoactivity of adrenal glands, which could have an impact on behavior (Lesage et al., 1996; Vodo et al., 2013; Vuong et al., 2010). Interestingly, female -but not male- rodents having undergone adrenalectomy and gonadectomy at birth showed a significant increase in estradiol in the cortex (Konkle and McCarthy, 2011). This suggests that the dimorphic changes seen in the PFC of offspring from morphine-treated dams could possibly be due to changes in reproductive hormones brought about by *in utero* morphine. In multiple brain regions, including the PFC, estradiol and testosterone change dynamically during rodent prepubertal and adolescent developmental periods

(Konkle and McCarthy, 2011). Therefore, using a maternal morphine paradigm that extends until PND 21 can lead to adverse outcomes selectively in female rodent offspring.

We assessed individual offspring behavior using a battery of behavioral tests, and then analyzed their behavioral phenotype holistically, using a composite score to evaluate possible resilient and/or vulnerable sub-populations. We found that adolescent female offspring from morphine-treated dams display increased baseline physical signs and increased total exploration time during the NORT training phase, compared to male offspring from morphine-treated dams. Interestingly, a higher percentage of female offspring from morphine-treated dams fell into the 'High' GBS category, suggesting that although they seemed "normal" when considering individual behaviors compared to female control offspring, they had a more severe overall behavioral phenotype in a critical developmental period. When their PFC transcriptomics were assessed, we also found significant changes in differentially expressed genes in female offspring from morphine-treated dams. Interestingly, carbonic anhydrase 13 (Car13) was highly upregulated in the PFC of these offspring, which to our knowledge is the first time it has been implicated in being altered in offspring after maternal opioid exposure. Carbonic anhydrases (CA) are important for many biological processes, including brain energy, metabolism, and pH homeostasis (Bueno-Junior et al., 2017; Deitmer et al., 2019). CA activity in the brain has been shown to be important for the consolidation of fear extinction memory in rodents, and a CA activator potentiated consolidation of extinction memory (Schmidt et al., 2020). Interestingly, studies have found that morphine exposure during gestation impaired offspring's ability to extinguish fear memories (Tan et al., 2015) and they required more time to extinguish drug-induced conditioned place

preference (Chiang et al., 2014; Shen et al., 2016) and drug self-administration reinstatement (Shen et al., 2016). Considering that CAs are found in oligodendrocytes and in brain regions abundant with myelin fibers (Haapasalo et al., 2020), future studies could further probe Car13 with regards to increased myelination.

We were also interested in investigating the effects of prenatal-preweaning morphine exposure on adult ethanol misuse using the I2BC paradigm, based on the literature on the interaction between ethanol and the opioid system. We were surprised not to find a significant difference in ethanol intake in offspring exposed to prenatal-preweaning morphine exposure, especially after one study investigating *in utero* methadone exposure found increased ethanol intake in adolescent male offspring (Grecco et al., 2022). This could be due to differences in the paradigms used to assess ethanol misuse (I2BC vs. drinking-in-the-dark), the opioid used during pregnancy, and the length of maternal drug exposure. Instead, we found that female offspring from morphine-treated dams display lower ethanol preference in the I2BC paradigm. Interestingly, female mice with a deletion of the preprodynorphin gene have decreased ethanol preference in a two-bottle choice experiment (Blednov et al., 2006), similar to the female offspring from morphine-treated dams in this study, suggesting that the change we observed could be due to alterations in the opioid receptor system following prenatal-preweaning morphine exposure. In addition, there is evidence showing that vulnerability to drug misuse is sex- and drug-dependent, and can be influenced by ovarian hormones (Carroll and Anker, 2010; Nicolas et al., 2022). More research is being published on vulnerable/susceptible populations and risk factors for drug misuse, like women who experienced childhood trauma and/or have mental health symptoms, for implementation of drug misuse prevention interventions (Kittirattanapaiboon et al., 2017; Pennington et

al., 2018; Shaffer et al., 2021; Surratt et al., 2018). For example, over 10 Tobacco Centers of Regulatory Science (TCORS) were established by the U.S. Food and Drug Administration to collect data on vulnerable populations for future tobacco regulation (Higgins et al., 2019). More preclinical studies are also examining the brain circuitry and molecular mechanisms that underlie vulnerable vs. resilient populations (Radke et al., 2021; Zhang et al., 2019). Future work is needed to determine if vulnerable and/or resilient sub-populations also exist among offspring exposed to prenatal-preweaning opioids that are exposed to commonly misused drugs, such as nicotine and ethanol, during adolescence and adulthood.

3.5. Conclusion

The data presented support the hypothesis that prenatal-preweaning morphine exposure alters offspring behavior. Although not modeled in the present study, important factors that influence and can exacerbate human offspring outcomes include: mother's poly-drug use, mother's concurrent psychiatric illnesses, and environmental/social stress experienced by the offspring. Questions left to be answered include whether stress can alter adolescent and adult drug reward sensitivity, and whether this confers susceptibility to drug use later in life. Probing potential targets mechanistically in vulnerable and resilient sub-populations of offspring exposed to *in utero* opioids is needed to develop novel therapeutic interventions early in development.

3.6. Figures

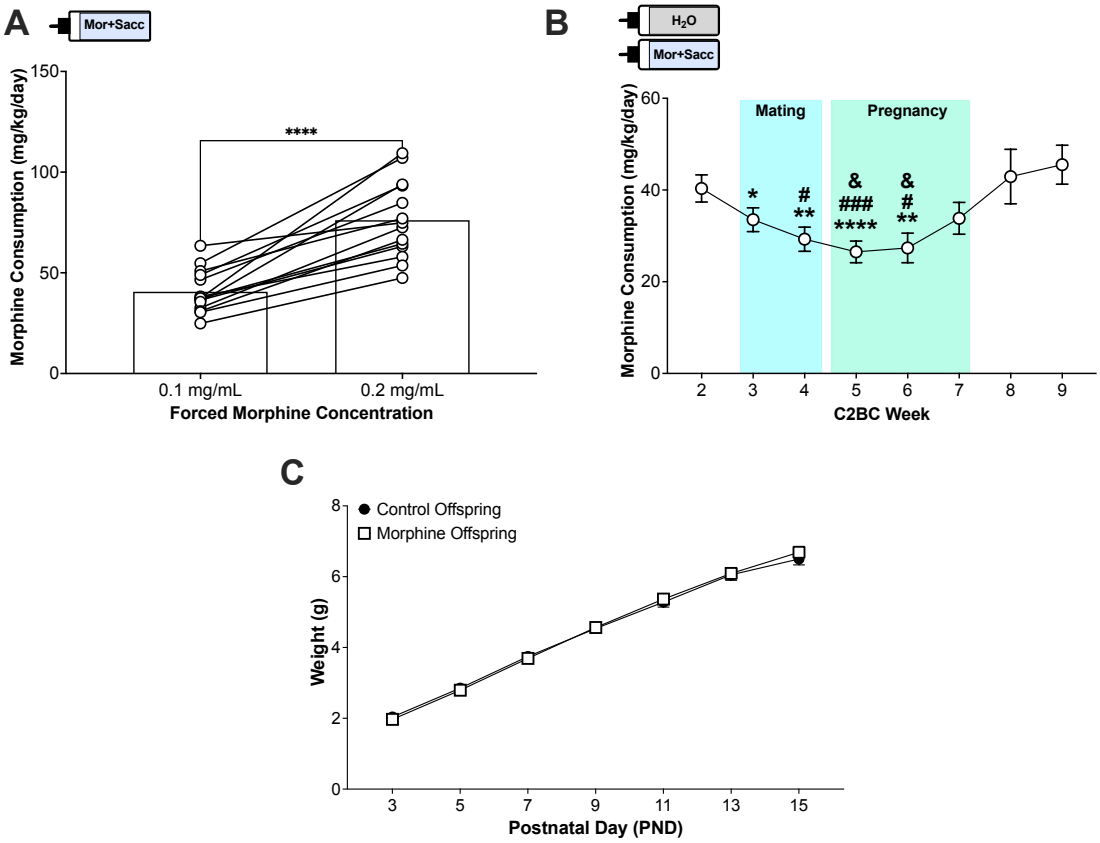


Figure 3-1: Morphine consumption for dams during oral morphine exposure and offspring bodyweight.

(**A & B**) Morphine consumption measured in female breeders while having access to a single bottle containing morphine in saccharin solution (forced morphine exposure) and two bottles containing morphine in saccharin solution vs. water (continuous two bottle choice). (**A**) Forced morphine consumption during week 1 of paradigm, when morphine dams' solution is ramped up from 0.1 mg/mL morphine to 0.2 mg/mL morphine, respectively. (n=14) **** p<0.0001 (**B**) Morphine intake in the C2BC paradigm during weeks 2-9. (n=14) * p<0.05, **p<0.01, **** p<0.0001 compared to Week 2; # p<0.05, ### p<0.001 compared to Week 3; & p<0.05 compared to Week 9. (**C**) Offspring body weight from PND 3-15 (litter n=23,20). Main effect of PND (RM mixed effects model; $F(1.349, 52.38)= 2150$, $p = <0.0001$).

Mor= morphine, Sacc= saccharin, C2BC= continuous two-bottle choice

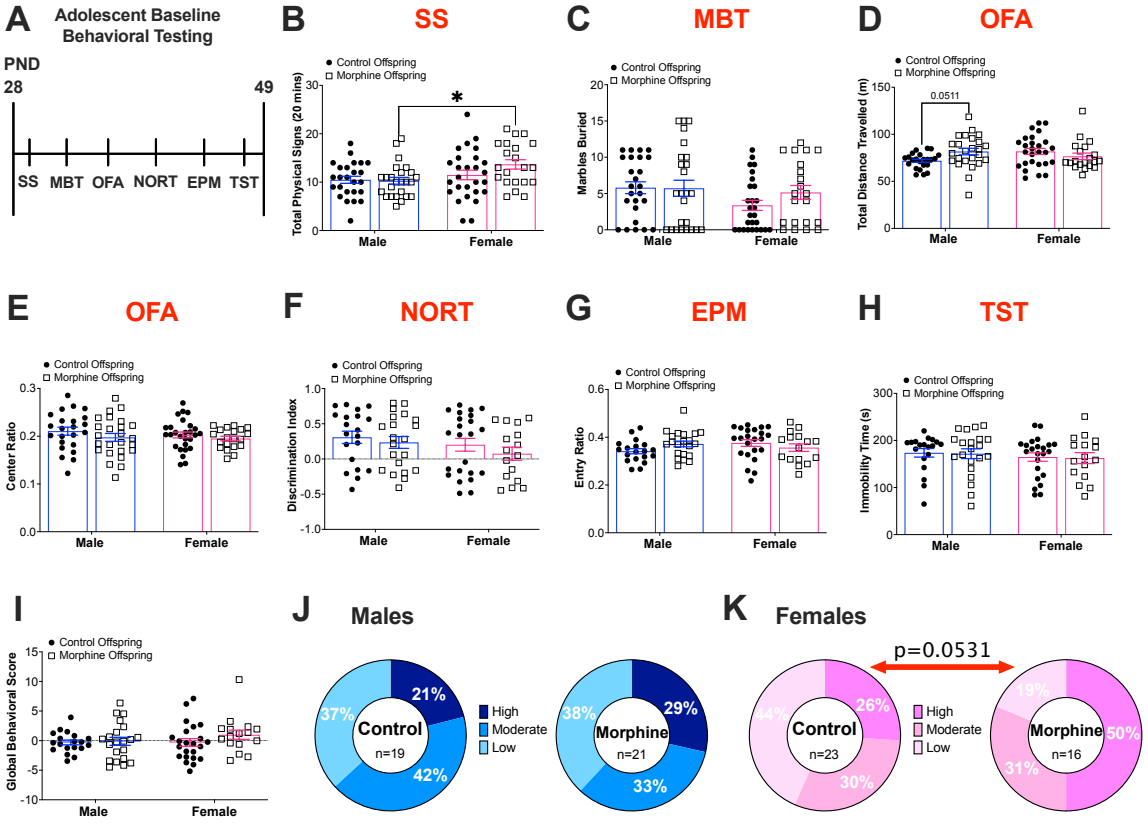


Figure 3-2: Sex-specific changes in baseline behavior in adolescent offspring from morphine-treated dams.

(**A**) Experimental timeline depicting the order of behavioral tests for PND 28-49 adolescent offspring. (**B**) Average number of total physical signs in control and morphine-treated offspring (control: n=25,27; morphine: n=25,21). (**C**) Average number of marbles buried in the Marble Burying Test (MBT) (control n=25,27; morphine: n=25,21). (**D**) Average distance travelled (m) in the Open Field Arena (OFA) (control: n=23,27; morphine: n=25,20). (**E**) Average center distance ratio in the OFA (control: n=23,27; morphine: n=25,20). (**F**) Average discrimination index in the Novel Object Recognition Test (NORT) (control: n=20,23; morphine: n=21,16). (**G**) Average open arm entry ratio in the Elevated Plus Maze (EPM) (control: n=19,23; morphine: n=21,16). (**H**) Average immobility time (s) in the Tail Suspension Test (TST) (control: n=19,23; morphine: n=21,16). (**I**) Average global behavioral score (GBS) calculated as the summation of the normalized behavioral data for each animal (control: n=19,23; morphine: n=21,16). Percent of male (**J**) and female **(K**) offspring from control and morphine-treated dams that classified as high, moderate, and low scorers based on their baseline behavior GBS. *p<0.05

PND = postnatal day; SS = somatic signs

sample size for control and morphine-treated offspring depicted as: n=male, female

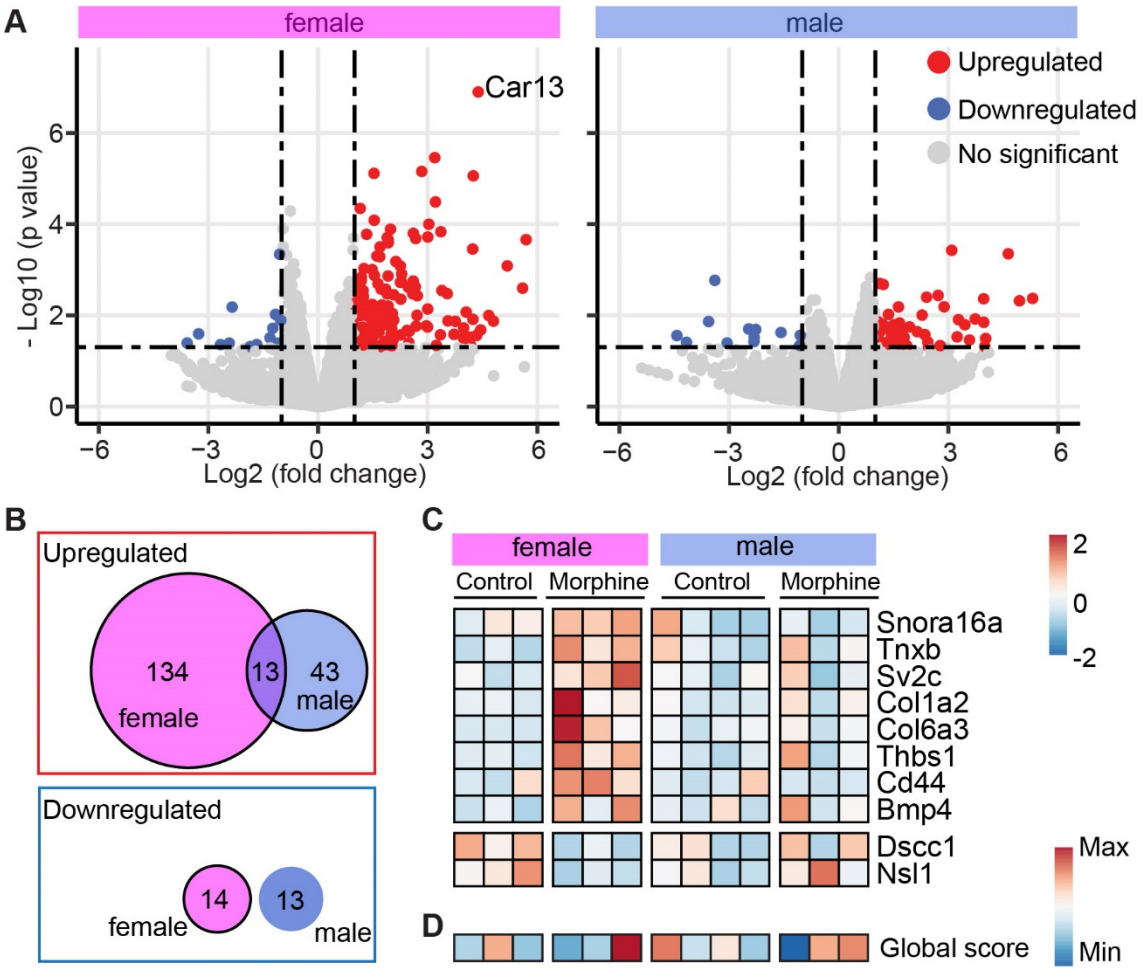


Figure 3-3: Transcriptomic changes under prenatal morphine exposure in male and female offspring.

(**A**) Volcano plots show the gene expression changes under morphine exposure in female (left panel) and male (right panel) offspring. Significantly upregulated (red) and downregulated (blue) genes are highlighted using cutoff: $p < 0.05$; fold changes >2. (**B**) Venn diagrams show the overlapping of significantly upregulated and downregulated genes under morphine exposure between male and female mice. (**C**) A heatmap shows normalized expression of selective differential expression genes in each sample. The color key for the heat map shows red as increased expression and blue as decreased expression normalized within each gene. (**D**) A heatmap shows the behavior global score of each sample.

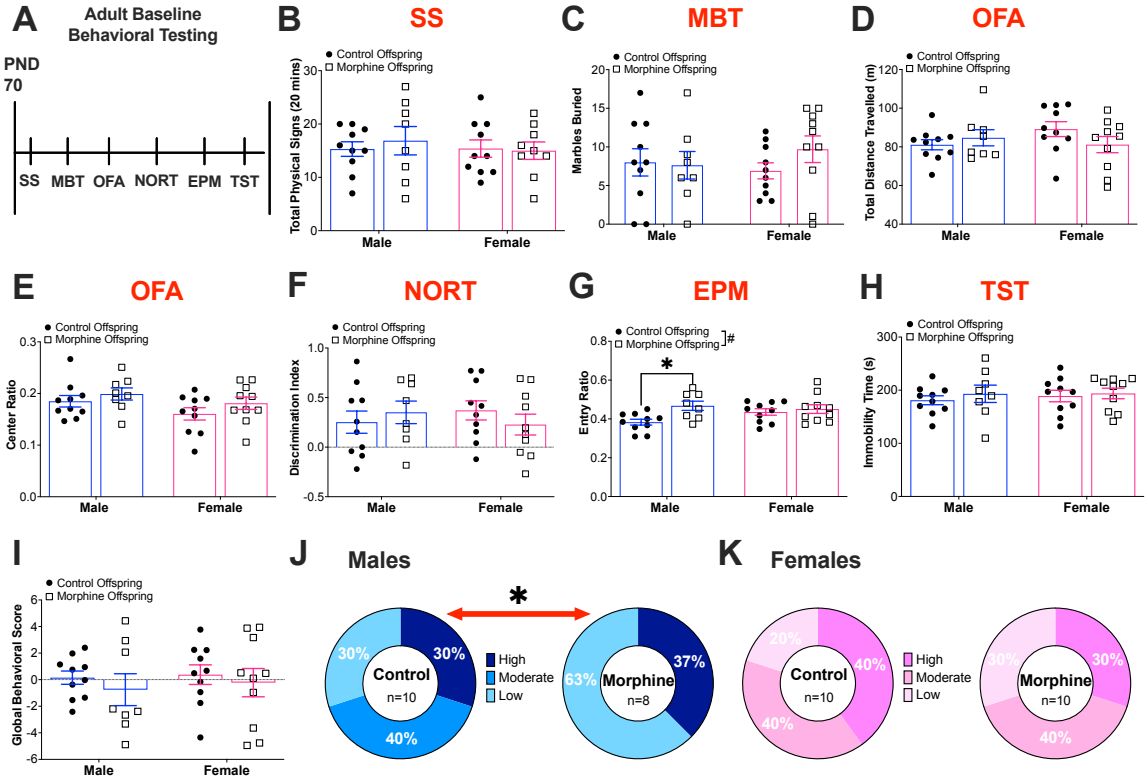


Figure 3-4: Sex-specific changes in baseline behavior in adult offspring from morphine-treated dams.

(**A**) Experimental timeline depicting the order of behavioral tests for adult (~2 months of age) offspring. (**B**) Average number of total physical signs in control and morphine-treated offspring (control: n=10,10; morphine: n=8,9). (**C**) Average number of marbles buried in the Marble Burying Test (MBT) (control: n=10,10; morphine: n=8,10). (**D**) Average distance travelled (m) in the Open Field Arena (OFA) (control: n=10,10; morphine: n=8,10). (**E**) Average center distance ratio in the OFA (control: n=10,10; morphine: n=8,10). (**F**) Average discrimination index in the Novel Object Recognition Test (NORT) (control: n=10,10; morphine: n=8,10). (**G**) Average open arm entry ratio in the Elevated Plus Maze (EPM) (control: n=10,10; morphine: n=8,10). (**H**) Average immobility time (s) in the Tail Suspension Test (TST) (control: n=10,10; morphine: n=8,10). (**I**) Average global behavioral score (GBS) calculated as the summation of the normalized behavioral data for each animal (control: n=10,10; morphine: n=8,10). Percent of male (**J**) and female (**K**) offspring from control and morphine-treated dams that classified as high, moderate, and low scorers based on their baseline behavior GBS.

#p<0.05 main effect; *p<0.05

PND = postnatal day; SS = somatic signs

sample size for control and morphine-treated offspring depicted as: n=male,female

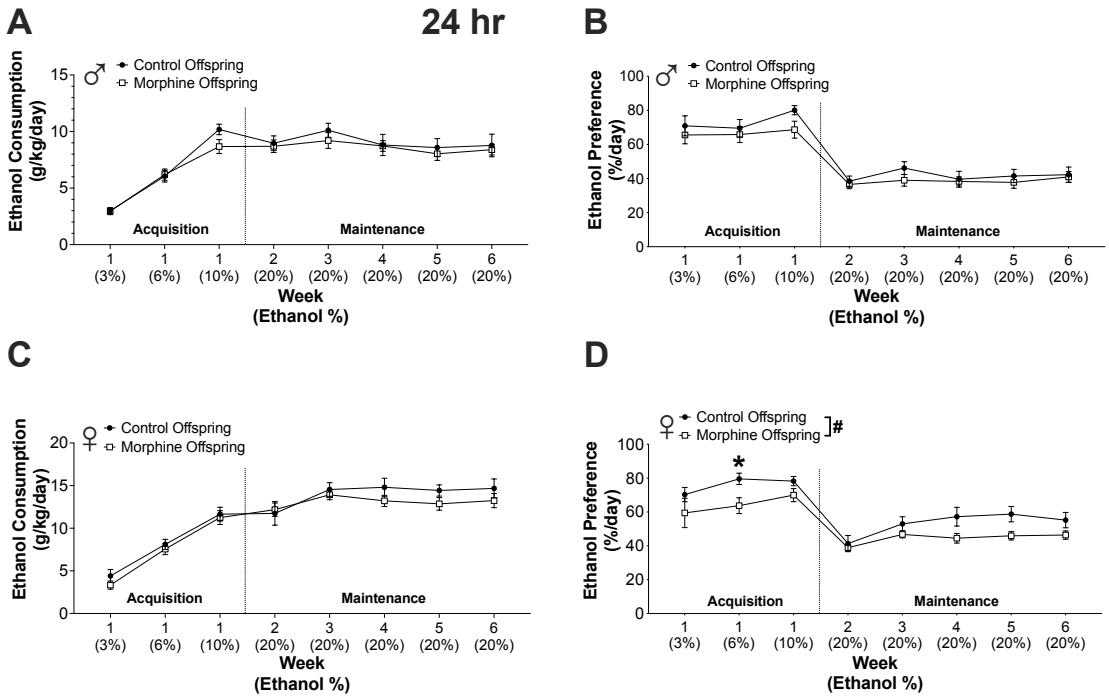


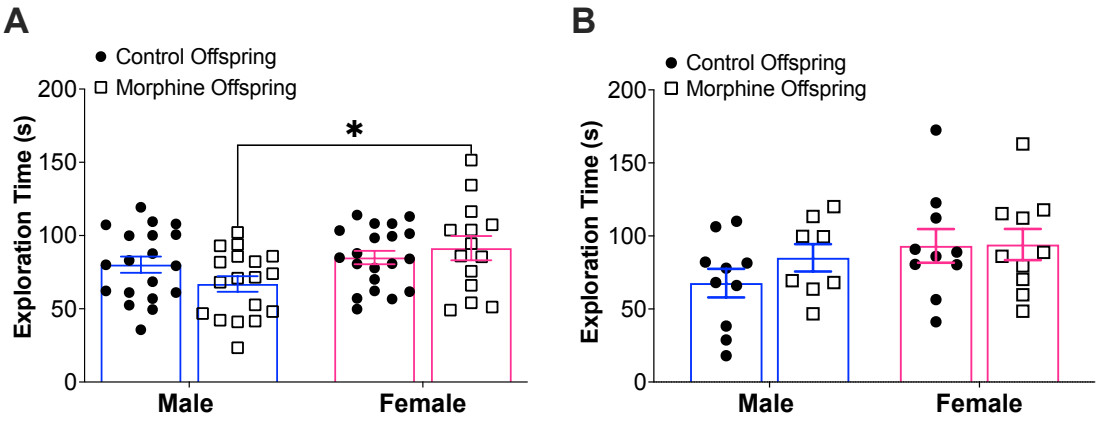
Figure 3-5: Ethanol intake and preference (24-hour) for male and female offspring in the I2BC paradigm. (A & B) Average 24-h ethanol intake (g/kg) (**A**) and average 24-h ethanol preference (%) (**B**) for male offspring during weeks 1-6 of I2BC paradigm (control n=12; morphine n=16). (**C & D)** Average 24-h ethanol intake (g/kg) (**C**) and ethanol preference (%) (**D**) for female offspring during weeks 1-6 of I2BC paradigm (control n=11; morphine n=12).

(**A**) Main effect of concentration for the 'Acquisition' phase (RM Mixed-Effects Model; $F(1.934,49.32) = 153.70$; $p < 0.0001$) and week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(3.180, 82.67) = 3.586$; $p = 0.0154$). (**B**) Main effect of week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(3.463, 90.05) = 3.105$; $p = 0.0245$). (**C**) Main effect of concentration for the 'Acquisition' phase (RM Mixed-Effects Model; $F(1.757,33.38) = 85.62$; $p < 0.0001$) and main effect of week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(2.718, 57.09) = 4.622$; $p = 0.0073$). (**D**) Main effect of week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(2.606, 54.73) = 11.87$; $p < 0.0001$).

# p<0.05 main effect of dam treatment for 'Acquisition' phase; * indicates post-hoc significance: * p<0.05

I2BC = intermittent two-bottle choice

3.7. Supplemental Figures

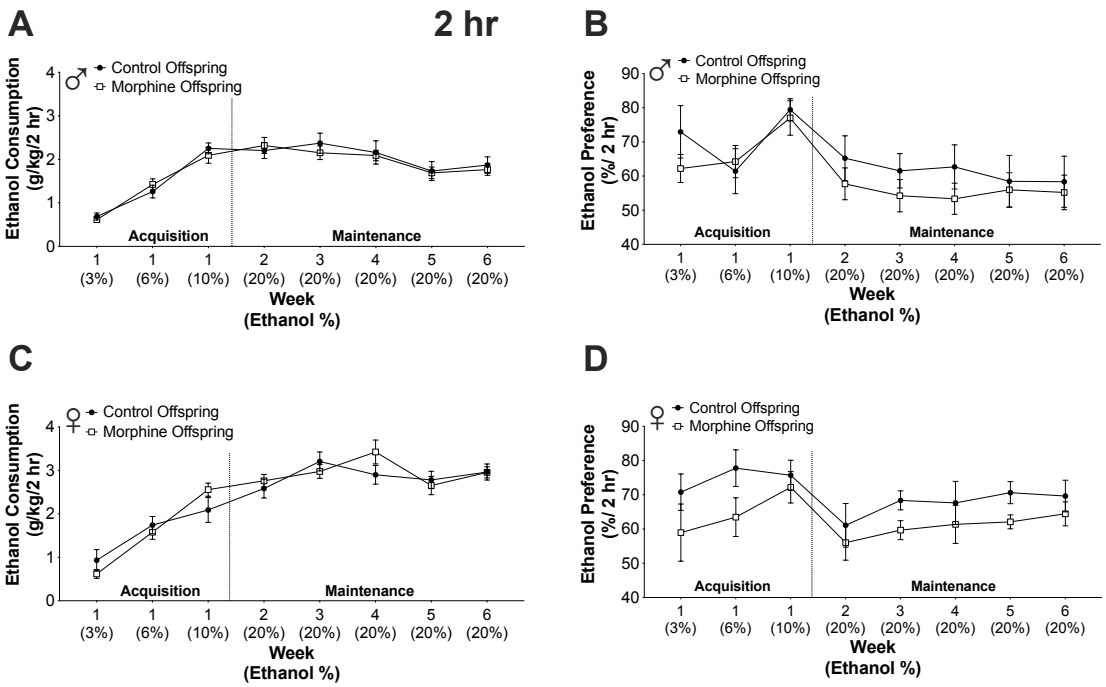


Supplemental Figure 3-1: Average exploration time for both objects in the training phase of the Novel Object Recognition Test (NORT) for adolescent

(**A**) (control: n=19,20; morphine: n=8,10) and adult (**B**) (control: n=10,10; morphine: n=18,14) offspring from control and morphine-treated dams.

sample size for control and morphine-treated offspring depicted as: n=male,female

* p<0.05

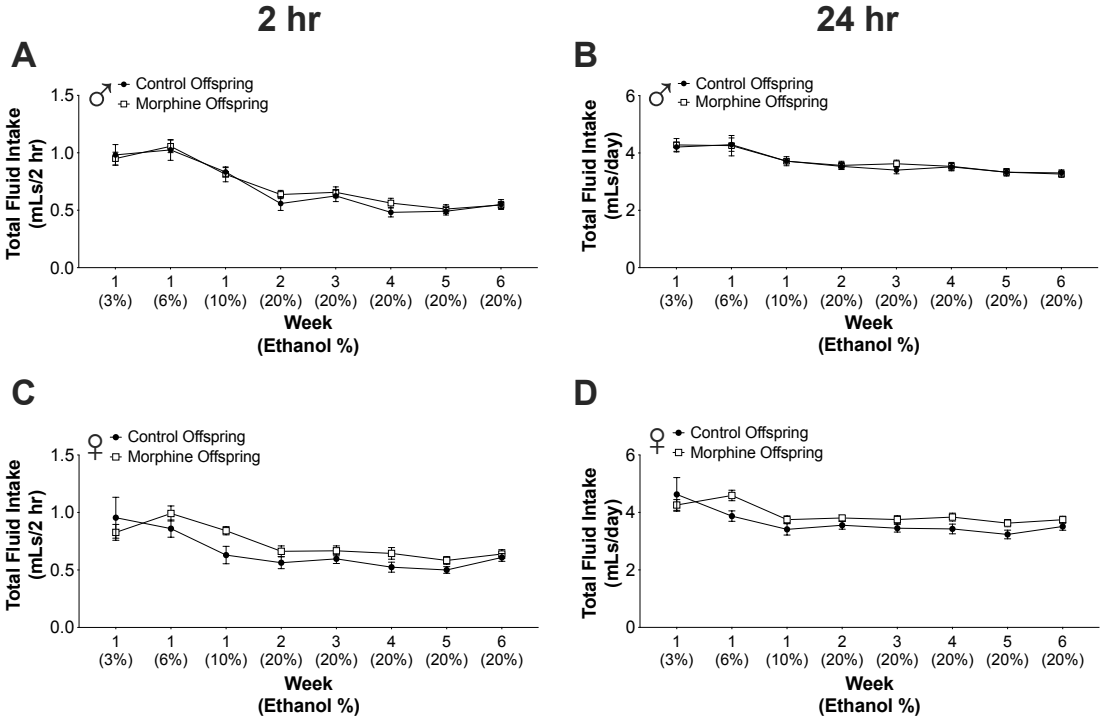


Supplemental Figure 3-2: Ethanol intake and preference (2-hour) for male and female offspring in the I2BC paradigm.

(A & B) Average 2-h ethanol intake (g/kg) (**A**) and average 2-h ethanol preference (%) (**B**) for male offspring during weeks 1-6 of I2BC paradigm (control n=12; morphine n=16). (**C & D**) Average 2-h ethanol intake (g/kg) (**C**) and ethanol preference (%) (**D**) for female offspring during weeks 1-6 of I2BC paradigm (control n=11; morphine n=12).

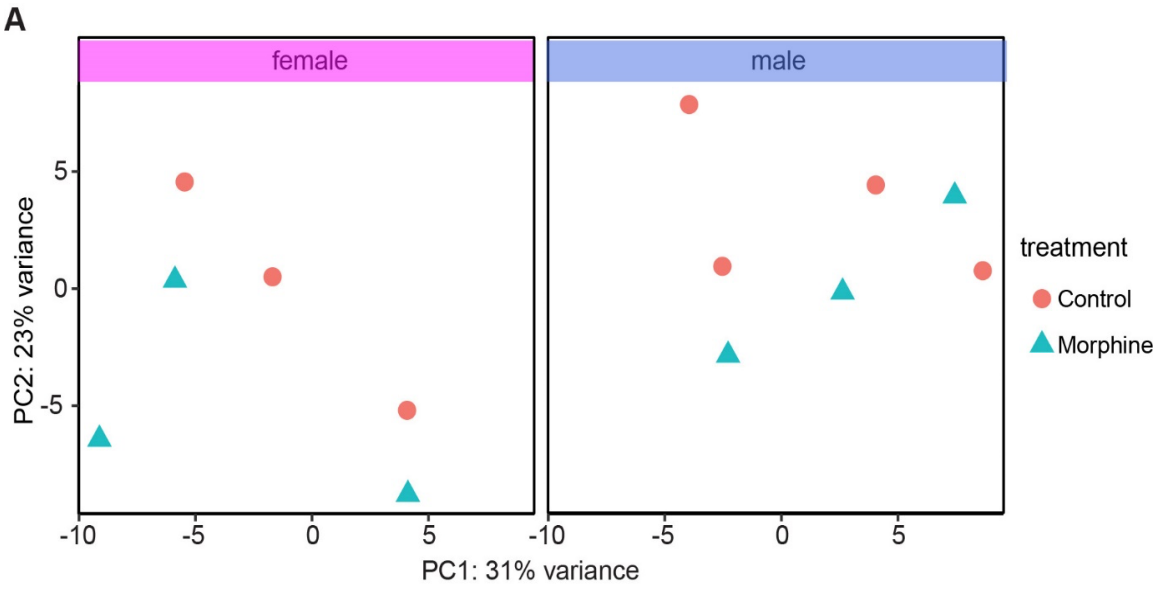
(**A**) Main effect of concentration for the 'Acquisition' phase (RM Mixed-Effects Model; $F(1.662,63.99) = 66.94$; $p < 0.0001$) and a main effect of week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(3.217, 83.64) = 7.922$; $p < 0.0001$). (**B**) Main effect of concentration for the 'Acquisition' phase (RM Mixed-Effects Model; $F(1.625,41.45) = 5.152$; $p = 0.0146$). (**C**) Main effect of concentration for the 'Acquisition' phase (RM Mixed-Effects Model; $F(1.891,36.87) = 36.15$; $p < 0.0001$) and a main effect of week for the 'Maintenance' phase (RM Two-Way ANOVA; $F(3.198, 67.16) = 3.423$; $p = 0.0198$).

I2BC = intermittent two-bottle choice



Supplemental Figure 3-3: Total fluid intake (2- and 24- hour) for offspring in the I2BC paradigm.

(**A**) Average 2-hour total fluid intake (mL) for male offspring from weeks 1-6 (control n=12; morphine n=16). (**B**) Average 24-hour total intake (ml) for male offspring from weeks 1-6 (control n=12; morphine n=16). (**C**) Average 2-hour total fluid intake for female offspring from weeks 1-6 (control n=11; morphine n=12). (**D**) Average 24-hour total fluid intake for female offspring from weeks 1-6 (control n=11; morphine n=12).



Supplemental Figure 3-4: Transcriptomic changes under prenatal morphine exposure in male and female offspring.

A principal component analysis shows separation between different conditions in different color (Morphine in red and Control in green) and sex in different panel (left panel: female; right panel: male).

3.8. Tables

Table 3-1: Significantly upregulated KEGG Pathway term analysis for Prefrontal Cortex (PFC) in female offspring

KEGG Pathway Term	Genes Associated with KEGG Pathway	Fold Change	p-value
ECM-receptor interaction	Tnxb, Sv2c, Col1a2, Itgb4, Fn1, Spp1, Col6a3, Thbs2, Thbs1, Cd44	16.45344575	6.30E-09
Focal adhesion	Tnxb, Col1a2, Itgb4, Pdgfd, Fn1, Spp1, Col6a3, Thbs2, Thbs1	6.483148772	5.85E-05
Protein digestion and absorption	Kcnk5, Col3a1, Col1a2, Kcnj13, Col8a2, Col6a3, Col8a1	9.384557945	8.56E-05
Phagosome	Mrc2, Msr1, H2-eb1, Mrc1, Thbs2, H2-aa, Thbs1, H2-q1	6.364409784	2.17E-04
Human papillomavirus infection	Tnxb, Col1a2, Itgb4, Fzd7, Fn1, Spp1, Col6a3, Thbs2, Thbs1, H2-q1	3.999732668	6.91E-04
Tuberculosis	Mrc2, Cd74, H2-eb1, Sphk1, Mrc1, Lbp, H2-aa	5.630734767	0.001330 72
PI3K-Akt signaling pathway	Tnxb, Col1a2, Itgb4, Pdgfd, Fn1, Spp1, Col6a3, Thbs2, Thbs1	3.629840956	0.002790 79
TGF-beta signaling pathway	Bmp4, Thbs1, Bmp6, Thsd4, Bmp5	7.620543294	0.003874 92
Amoebiasis	Col3a1, Col1a2, Serpinb6b, Serpinb6c, Fn1	6.765902924	0.005913 56
Hippo signaling pathway	Bmp4, Cdh1, Fzd7, Bmp6, Bmp5	4.61115677	0.021735 99
Antigen processing and presentation	Cd74, H2-eb1, H2-aa, H2-q1	6.435125448	0.023090 19
Hematopoietic cell lineage	H2-eb1, Anpep, H2-aa, Cd44	6.161290323	0.025850 1
AGE-RAGE signaling pathway in diabetic complications	Thbd, Col3a1, Col1a2, Fn1	5.734270201	0.031089 08
Cytokine-cytokine receptor interaction	Bmp4, Il22ra1, Lepr, Tnfrsf11b, Bmp6, Bmp5	2.975143615	0.047681 43

Abbreviations:

KEGG = Kyoto Encyclopedia of Genes and Genomes

ECM = Extracellular Matrix

3.9. Supplemental Tables

Supplemental Table 3-1: Significantly upregulated Differentially Expressed Genes (DEGs) with a +/- 1-fold change in the Prefrontal Cortex (PFC) in female offspring

DEG	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Dscc1	2.45416386	-3.579886467	1.7471662	-2.04896733	0.04046531	0.9997776
Mir7052	3.8883574	-3.266789729	1.46209686	-2.23431827	0.02546214	0.9997776
Nsl1	3.8404023	-2.66929364	1.32453141	-2.01527394	0.04387595	0.9997776
Iqca	4.50143017	-2.427800219	1.18576892	-2.04744802	0.04061412	0.9997776
Xlr3a	12.3842424	-2.350741257	0.86517261	-2.71707776	0.00658611	0.9997776
Gm10635	13.9386863	-1.853957415	0.93879432	-1.97482812	0.04828766	0.9997776
Lars2	13658.4426	-1.676781892	0.8308683	-2.01810791	0.04358002	0.9997776
Nrtn	39.8848674	-1.315908292	0.6105491	-2.15528662	0.03113941	0.9997776
B930059L03Rik	23.8454128	-1.238584667	0.52918498	-2.34055145	0.01925529	0.9997776
1700040L02Rik	14.3935847	-1.216146423	0.6115058	-1.98877332	0.04672623	0.9997776
Urah	42.7327015	-1.163435474	0.44897176	-2.5913333	0.00956049	0.9997776
Adamts13	18.580417	-1.104179363	0.53947274	-2.04677508	0.04068017	0.9997776
Usmg5	705.735919	-1.052116245	0.30022834	-3.50438685	0.00045766	0.33087124
Tnnc1	159.531182	-1.020181964	0.41006413	-2.48785959	0.01285145	0.9997776
Samd9l	68.699348	1.015058944	0.36671429	2.76798306	0.00564044	0.9997776
Anxa4	50.3470805	1.016016091	0.359658	2.824950592	0.00472879	0.9709457
Ptpn14	74.0327011	1.021900665	0.37562074	2.72056509	0.00651704	0.9997776
Nt5e	48.0805731	1.026086225	0.40149769	2.555646621	0.01059907	0.9997776
Acan	35.0497522	1.032225903	0.43463019	2.374952177	0.01755122	0.9997776
Arhgef5	26.3544265	1.053494646	0.5217476	2.019165294	0.04347004	0.9997776
Cd44	26.7573548	1.067975504	0.48944931	2.181994104	0.02910997	0.9997776
Mxra8	45.3136186	1.072268873	0.52185354	2.054731418	0.03990495	0.9997776
Cp	182.737576	1.082565036	0.34639643	3.125219949	0.00177672	0.66923197

Thbs2	94.8130745	1.08559277	0.38948707	2.787237021	0.00531596	0.9997776
Perp	70.9645296	1.095214291	0.38721822	2.828416205	0.0046779	0.9709457
Zic1	133.454915	1.099348503	0.36695499	2.995867406	0.00273665	0.80938616
Bmp4	33.2847097	1.124809522	0.51102705	2.201076296	0.02773062	0.9997776
Fzd7	63.1289172	1.131894052	0.40999207	2.760770579	0.00576652	0.9997776
Tnxb	37.2981512	1.133867372	0.42046175	2.696719419	0.00700263	0.9997776
Car14	28.8346152	1.145206365	0.56737094	2.018443815	0.04354506	0.9997776
Sv2c	37.5966028	1.151664607	0.44828207	2.569062409	0.01019741	0.9997776
Itgb4	49.4146138	1.153673475	0.44502166	2.592398474	0.00953093	0.9997776
Ahnak	290.694181	1.154114894	0.28296221	4.078689197	4.53E-05	0.12527766
P2ry1	20.7757542	1.180425942	0.57193334	2.063922253	0.03902509	0.9997776
Mrc1	60.0224043	1.181481102	0.37767145	3.128330436	0.00175802	0.66923197
Pdgfd	45.8925223	1.187671163	0.37305587	3.183628161	0.00145442	0.60404728
Foxc1	87.6382394	1.190147706	0.45509206	2.615180092	0.00891804	0.9997776
Tspan18	23.5999602	1.19819907	0.56874351	2.106747688	0.03513946	0.9997776
Myof	46.0534555	1.224993511	0.46927711	2.610384093	0.00904406	0.9997776
Plekha4	26.9141226	1.226295023	0.51928251	2.361518075	0.01820029	0.9997776
Bgn	110.224789	1.23352045	0.41376563	2.981205696	0.00287116	0.82419144
Tfap2b	20.2525569	1.240174688	0.61770509	2.007713252	0.04467377	0.9997776
Slc13a3	131.124455	1.243283083	0.43059488	2.887361521	0.00388488	0.88031502
Pgm5	56.773649	1.24876005	0.46105029	2.708511558	0.00675858	0.9997776
Col1a2	279.216896	1.255258905	0.37951804	3.307507863	0.0009413	0.52497684
Adamts9	19.8022566	1.255707453	0.55470831	2.263725675	0.02359099	0.9997776
Slc35g1	28.0525268	1.272608399	0.61174738	2.080284197	0.03749947	0.9997776
Crispld2	18.1134626	1.295861862	0.59507545	2.177642947	0.02943263	0.9997776
Tbx18	35.6107305	1.298016612	0.57049128	2.275261084	0.02289027	0.9997776
Sphk1	29.3505797	1.3063168	0.63925156	2.04350977	0.041002	0.9997776
Stra6	36.9223727	1.32708921	0.57586585	2.304511027	0.02119397	0.9997776
Cyp1b1	64.3903298	1.329763939	0.3533124	3.76370586	0.00016741	0.20462644
Col8a1	46.1201797	1.351527113	0.5267249	2.565907006	0.01029064	0.9997776

Asgr1	15.1629234	1.356839824	0.67313602	2.015699344	0.04383142	0.9997776
Col3a1	75.0303929	1.40285512	0.51163812	2.741889368	0.00610869	0.9997776
Aox3	34.996166	1.40360858	0.59822244	2.346298762	0.0189609	0.9997776
Ano1	15.8226049	1.453877166	0.61733362	2.355091493	0.01851815	0.9997776
Slc16a9	26.72924	1.47903704	0.60830754	2.431396844	0.01504073	0.9997776
Sned1	55.5575242	1.481437917	0.45004854	3.291729167	0.00099574	0.53990939
Serpind1	21.9093717	1.482279425	0.53656263	2.762546895	0.00573523	0.9997776
Slc22a6	47.138362	1.491333632	0.58850742	2.534094864	0.01127382	0.9997776
Slc26a2	48.3057922	1.499195555	0.55465745	2.702921504	0.0068733	0.9997776
Slco2a1	12.4081992	1.51066821	0.69278225	2.180581574	0.02921438	0.9997776
Rec114	9.70848152	1.515274991	0.75192482	2.015194801	0.04388424	0.9997776
Phldb2	114.788651	1.530986994	0.34228569	4.472833743	7.72E-06	0.0341441
Lepr	74.6007369	1.53731421	0.39037682	3.938026363	8.22E-05	0.17818413
Anxa2	99.6776172	1.540306123	0.47982073	3.210170032	0.00132657	0.57543432
Emilin1	29.8811919	1.545246349	0.61734433	2.50305426	0.01231267	0.9997776
Thsd4	83.076273	1.611387944	0.4630748	3.479757352	0.00050187	0.33687901
Scml2	27.9476987	1.612093792	0.59720255	2.699408752	0.00694628	0.9997776
Zic4	33.0757344	1.637426935	0.53054277	3.086324116	0.00202648	0.70276366
Tbx15	17.3112773	1.663473332	0.73649739	2.258627615	0.02390656	0.9997776
Cped1	52.4676951	1.688129814	0.48629598	3.471404022	0.00051774	0.33687901
C1qtnf7	7.87310814	1.693058416	0.85556015	1.978888816	0.04782853	0.9997776
Fgl2	31.9139547	1.697484288	0.47122556	3.602275503	0.00031544	0.25656095
Lox	24.7267883	1.717419854	0.69463213	2.472416373	0.01342031	0.9997776
Col6a3	21.424507	1.725861233	0.57444925	3.004375474	0.00266127	0.79957904
Snora16a	6.88489369	1.738630177	0.88263328	1.969821694	0.04885881	0.9997776
Tnfrsf11b	30.6116519	1.742867794	0.66990015	2.601682934	0.00927676	0.9997776
Anpep	37.5733383	1.743181829	0.66684149	2.614087236	0.00894662	0.9997776
Cd74	28.0405215	1.744719562	0.63415209	2.751263608	0.00593659	0.9997776
Kcne4	12.0613138	1.751514168	0.7326989	2.39049652	0.01682561	0.9997776
Alx4	24.2571539	1.759296317	0.71950184	2.445158902	0.01447883	0.9997776

Aebp1	136.712092	1.782607352	0.90082557	1.978859638	0.04783181	0.9997776
Apobr	7.86683154	1.794355939	0.87467129	2.051463178	0.04022186	0.9997776
Crabp2	22.1810067	1.816289695	0.72888007	2.491891027	0.0127065	0.9997776
Slc6a20a	62.2129723	1.832220267	0.90839471	2.016986941	0.04369688	0.9997776
Cubn	8.46679955	1.835286696	0.88427394	2.075473023	0.03794272	0.9997776
Serpinb6b	23.2464137	1.847775759	0.61505786	3.004230795	0.00266253	0.79957904
Kcnj13	10.1589984	1.876644972	0.94699506	1.981684011	0.04751462	0.9997776
Sema3b	22.1426607	1.890275184	0.64504956	2.930434053	0.00338489	0.87240747
Thbs1	28.6494182	1.893349394	0.50871183	3.721850499	0.00019777	0.20462644
Tgfbi	29.9422404	1.915037122	0.59835225	3.200517969	0.00137181	0.58212381
Fgfbp1	20.8884353	1.915870614	0.73325798	2.612819301	0.00897988	0.9997776
Slc12a7	49.4270736	1.919200339	0.51944599	3.69470624	0.00022014	0.20462644
Aim1	12.430347	1.919753979	0.70214305	2.734135128	0.00625444	0.9997776
F13a1	38.1533269	1.920639907	0.52539093	3.655639662	0.00025654	0.22762227
Themis2	10.6465541	1.931932402	0.78112646	2.47326458	0.0133885	0.9997776
Mrgprf	9.15570307	1.971043144	0.94209085	2.092200712	0.03642057	0.9997776
Thbd	233.225935	1.977245826	0.83625919	2.364393549	0.01805962	0.9997776
Bmp6	55.3757746	1.98203354	0.51781887	3.827658015	0.00012937	0.20462644
Fn1	544.519077	1.991303817	0.8996625	2.213389819	0.02687078	0.9997776
Mrc2	105.405356	2.001090939	0.81220658	2.463770898	0.0137484	0.9997776
Wfikkn2	11.3775682	2.014711692	0.85841848	2.347004106	0.01892505	0.9997776
Tnk2os	5.46244172	2.018263477	1.01167053	1.994980989	0.04604495	0.9997776
1500015O10Rik	14.69476	2.033314847	0.77983201	2.607375477	0.00912393	0.9997776
Acss3	6.36224404	2.047117199	0.97484913	2.099932331	0.03573479	0.9997776
Slc9a2	27.4540619	2.068834649	0.70920317	2.917125511	0.00353274	0.87240747
Aoc3	9.51860849	2.06904245	0.83049161	2.491346594	0.01272599	0.9997776
Ston1	20.9109141	2.12972959	0.62542666	3.405242751	0.00066105	0.41624991
Fam180a	25.2058156	2.252187689	0.72509933	3.106040226	0.00189611	0.68540836
Foxd1	32.5180713	2.252983484	0.67419451	3.341741053	0.00083255	0.47797987
Gm4951	6.20885821	2.253633006	1.01901856	2.211572089	0.02699625	0.9997776

H2-Eb1	12.7545825	2.26213238	0.79492701	2.845710805	0.00443124	0.96108712
Tspan11	22.4725512	2.276092991	0.72853233	3.124216862	0.00178279	0.66923197
1700024P16Rik	24.75991	2.288538572	0.70843573	3.230411012	0.00123612	0.57543432
1700020A23Rik	6.11875478	2.437035038	1.0587159	2.301878185	0.02134204	0.9997776
Eya2	18.3888572	2.469690501	0.8144888	3.032197015	0.00242781	0.76436759
Cnksr1	4.09900606	2.485976737	1.20852154	2.057039664	0.03968241	0.9997776
Vnn1	5.33485603	2.496003437	1.09220961	2.285278764	0.02229649	0.9997776
Cd109	9.28807738	2.591419109	0.88384306	2.931990101	0.00336798	0.87240747
Frk	9.41768415	2.605028696	0.83344913	3.125600125	0.00177443	0.66923197
Col8a2	32.9104608	2.607456658	0.69056499	3.775830947	0.00015948	0.20462644
Lbp	18.6419336	2.616312693	0.85016909	3.077402732	0.00208813	0.70276366
Bche	96.4008425	2.660732386	0.87592274	3.037633633	0.00238444	0.7630197
Bmp5	16.2935408	2.672735784	0.72041651	3.709986837	0.00020727	0.20462644
Ogn	219.199972	2.682139417	1.12594966	2.382113074	0.01721361	0.9997776
Kdelr3	6.15431716	2.70766587	1.05098907	2.576302608	0.00998632	0.9997776
Spp1	74.4229086	2.71490407	0.9356812	2.901526793	0.00371349	0.88031502
H2-Aa	25.2216386	2.836564514	0.63120544	4.493884801	6.99E-06	0.0341441
Tspan8	7.82329142	2.963257686	1.24107234	2.38765911	0.01695606	0.9997776
Prg4	59.0753609	2.99566592	1.26649112	2.365327221	0.01801415	0.9997776
Kcnk5	6.46609036	3.000117809	1.11773553	2.684103462	0.00727246	0.9997776
Msln	15.2286437	3.002976818	0.80516222	3.729654425	0.00019174	0.20462644
Ptgdr	17.0847344	3.030709683	0.77932218	3.888904716	0.0001007	0.19656179
Omd	34.7396393	3.193209987	0.68819329	4.63999003	3.48E-06	0.03400638
Adamtsl3	24.742065	3.212081849	0.77322761	4.154122046	3.27E-05	0.10623398
Dapl1	2.85923425	3.236631653	1.61854414	1.999717878	0.04553074	0.9997776
Serpinb6c	3.72451837	3.345959166	1.51034117	2.21536646	0.02673492	0.9997776
Slc26a7	16.9829971	3.358623384	0.88473657	3.796184671	0.00014694	0.20462644
Piezo2	6.94187286	3.388595088	1.13632664	2.982060757	0.00286315	0.82419144
Prdm6	7.85991555	3.534688256	1.20471613	2.934042445	0.00334579	0.87240747
H2-Q1	5.28054171	3.555009506	1.43544077	2.476597838	0.01326413	0.9997776

Kdf1	3.85653318	3.718230141	1.67187853	2.223983421	0.02614956	0.9997776
Six1	4.83637457	3.754884653	1.52036827	2.469720487	0.01352187	0.9997776
Rubie	2.81674722	3.976216152	1.84552143	2.154521802	0.03119927	0.9997776
Msr1	2.82942526	3.981339565	1.69592318	2.347594285	0.01889509	0.9997776
Vsx1	5.15222819	4.053619755	1.54046858	2.63141996	0.00850289	0.9997776
C130021I20Rik	3.14482605	4.152892757	1.91391109	2.169846223	0.0300185	0.9997776
Glod5	1.56290509	4.169769953	2.07640082	2.008171989	0.04462502	0.9997776
Six2	11.2216807	4.230963563	1.18384709	3.573910505	0.00035169	0.27459891
Spp2	3.44684868	4.235832019	1.69070613	2.505362668	0.01223259	0.9997776
Foxc2	21.4192799	4.246522558	0.95512026	4.446060606	8.75E-06	0.0341441
Il22ra1	1.76229449	4.339810251	1.96922269	2.20381894	0.02753708	0.9997776
Car13	28.3726841	4.381946063	0.82934779	5.283604929	1.27E-07	0.00247253
4930509J09Rik	1.89446325	4.449583618	1.92604172	2.310221831	0.02087588	0.9997776
Scgn	4.451315	4.670419054	1.81519555	2.57295643	0.01008339	0.9997776
Six3os1	2.41379929	4.79849728	1.94042025	2.472916513	0.01340155	0.9997776
Slc47a1	52.5978205	5.176621612	1.54772233	3.344670747	0.0008238	0.47797987
Cdh1	25.5875364	5.59594521	1.85388871	3.018490366	0.00254038	0.78711295
Bnc2	8.95431245	5.692821072	1.54017411	3.696219175	0.00021883	0.20462644

Supplemental Table 3-2: Significantly upregulated Differentially Expressed Genes (DEGs) with a +/- 1-fold change in the Prefrontal Cortex (PFC) in male offspring

DEG	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Gm15412	2.39035725	-4.427369844	2.01151337	-2.20101437	0.02773501	0.99963383
Mir6935	1.98776973	-4.166691073	2.01820996	-2.06454787	0.0389658	0.99963383
Tdg	4.24914538	-3.561087967	1.44430504	-2.46560654	0.01367815	0.99963383
Capn11	10.7348555	-3.390259606	1.08079469	-3.13682111	0.0017079	0.99963383
2700070H01Rik	6.41867722	-3.053993444	1.49052643	-2.04893613	0.04046836	0.99963383
Rnf8-cmtr1	7.68984114	-2.463372637	1.05808919	-2.32813327	0.01990503	0.99963383
S1pr4	5.89206165	-2.319888477	1.11592838	-2.07888654	0.03762778	0.99963383
Nxph4	7.8890222	-2.314390991	1.06954395	-2.16390452	0.03047168	0.99963383
Xlr3b	6.001845	-2.270351033	0.979394	-2.31811817	0.0204429	0.99963383
Ttc39a	15.5100424	-1.578579052	0.69830033	-2.26060189	0.02378392	0.99963383
Hcrtr1	22.1861621	-1.090435462	0.55494209	-1.96495362	0.04941961	0.99963383
Spata9	18.8630169	-1.066368957	0.53338257	-1.99925722	0.04558053	0.99963383
Ntsr1	40.6402674	-1.051033923	0.47635723	-2.20639862	0.0273561	0.99963383
Col8a1	37.6375902	1.037270694	0.46703132	2.220987449	0.02635181	0.99963383
Phldb2	71.5274207	1.097008873	0.44705328	2.453866051	0.01413296	0.99963383
5730480H06Rik	19.9303807	1.097663229	0.51755309	2.120870771	0.03393268	0.99963383
Mxra8	23.9117504	1.117484859	0.53852396	2.075088476	0.03797834	0.99963383
4430402I18Rik	21.8881771	1.121545256	0.56080256	1.999893252	0.04551179	0.99963383
Ccdc173	60.8778535	1.122380817	0.36332847	3.089162905	0.00200721	0.99963383
Gm20754	26.5205597	1.139747931	0.4984053	2.286789356	0.02220812	0.99963383
Tbx18	31.3731345	1.197347141	0.49565818	2.415671089	0.01570624	0.99963383
Casp12	16.3785017	1.199202004	0.59180566	2.026344261	0.04272952	0.99963383
Pin4	70.0436157	1.218963625	0.39648921	3.074392921	0.00210931	0.99963383
Gmnc	23.5943621	1.265990909	0.63035894	2.008365136	0.04460451	0.99963383
Angptl4	15.9123051	1.269336895	0.53498021	2.372680095	0.01765955	0.99963383
Mir17hg	10.3301579	1.283783156	0.64850971	1.979589711	0.04774965	0.99963383

Cyp26b1	24.2165301	1.308379887	0.64894685	2.016158787	0.04378337	0.99963383
Zic4	21.0490764	1.328075833	0.64320459	2.064779795	0.03894384	0.99963383
Aebp1	73.3920967	1.359710195	0.52467546	2.591526195	0.00955513	0.99963383
Serpind1	12.1750964	1.404178704	0.693302	2.025349271	0.04283151	0.99963383
S100a4	8.28981918	1.435775874	0.72236944	1.987592224	0.04685681	0.99963383
Tnfrsf11b	19.4410406	1.442001867	0.63737029	2.262424033	0.02367122	0.99963383
Gpc3	17.7443635	1.443355168	0.66807079	2.160482388	0.03073535	0.99963383
Trpm6	15.8758778	1.453240535	0.65237262	2.227623429	0.02590564	0.99963383
AW822252	9.7425867	1.466550405	0.68113654	2.153093118	0.03131136	0.99963383
9430065F17Rik	13.7021277	1.503733042	0.62523367	2.405073661	0.01616921	0.99963383
Ajuba	9.74879153	1.518027257	0.69566481	2.182124545	0.02910034	0.99963383
Lrrc23	11.9661179	1.634366227	0.80671052	2.025963689	0.04276851	0.99963383
Rab26os	18.7405706	1.634681203	0.60115857	2.719218022	0.00654365	0.99963383
Angptl2	13.5385607	1.663254598	0.68184928	2.439328825	0.01471457	0.99963383
Abca4	15.4934417	1.701394334	0.7446426	2.284846884	0.02232181	0.99963383
Crabp2	14.2764388	1.727233187	0.7942658	2.174628684	0.02965795	0.99963383
Mylpf	6.57943197	1.74159713	0.82516456	2.110605838	0.03480621	0.99963383
Eya2	9.99010547	1.790577064	0.82719622	2.164634004	0.03041573	0.99963383
Gm8234	6.39086244	1.822996225	0.91194661	1.999016387	0.04560658	0.99963383
D4Ertd617e	6.50476173	1.863229701	0.93574391	1.991174804	0.04646167	0.99963383
Lbp	14.2694857	1.937189971	0.81838806	2.367079947	0.01792906	0.99963383
Frrs1	6.08501332	2.149172219	0.94419985	2.276183618	0.02283502	0.99963383
Rpph1	10.3378571	2.281159105	0.88434839	2.579480133	0.00989492	0.99963383
Fgfbp1	10.8254574	2.356335634	1.06208358	2.218597189	0.02651414	0.99963383
1500015O10Rik	18.8678327	2.402913754	0.83476246	2.878559903	0.00399495	0.99963383
Apoa1	4.79719665	2.437793533	1.17782293	2.069745343	0.0384762	0.99963383
Kl	44.8088788	2.454796727	1.2508144	1.962558738	0.04969747	0.99963383
Drc7	10.8792541	2.719693975	0.93577522	2.90635393	0.00365668	0.99963383
Tmprss11a	3.00421405	2.774772648	1.39117551	1.994552544	0.0460917	0.99963383
F5	10.034564	2.882836085	1.05925433	2.721571214	0.00649724	0.99963383

Trpv4	17.7823276	3.090648839	0.86893797	3.5568118	0.00037538	0.99963383
Hdc	2.59141331	3.239725482	1.49018711	2.174039392	0.02970218	0.99963383
Calml4	5.69244865	3.276364201	1.31095239	2.499224408	0.01244655	0.99963383
Krt18	4.43196854	3.449603729	1.43291606	2.407401126	0.01606651	0.99963383
Ifi27l2a	1.8104997	3.578089026	1.69466856	2.111379837	0.03473968	0.99963383
Clic6	19.1230105	3.733843562	1.48653877	2.511770055	0.01201273	0.99963383
Six3	3.72227799	3.964997402	1.39004286	2.852428161	0.00433866	0.99963383
Cldn2	3.57205421	3.967867554	1.61875107	2.451190696	0.01423845	0.99963383
Cdh3	1.36372159	3.996273115	2.02218514	1.976215255	0.0481304	0.99963383
Tmem72	2.30129661	4.021375856	1.87713927	2.142289563	0.03217019	0.99963383
Wdr86	7.60326777	4.637477253	1.32091422	3.510808786	0.00044675	0.99963383
Prl	2.56763593	4.94474821	1.75342613	2.820049352	0.00480163	0.99963383
Gh	12.9292206	5.304734365	1.8559425	2.858242848	0.00425994	0.99963383

CHAPTER 4: Adolescent Nicotine Use Increases Binge-like Ethanol Drinking in Females Exposed to Prenatal-Preweaning Morphine

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4.1. Introduction

The rates of opioid use have increased drastically among all populations, including pregnant women (Clemans-Cope et al., 2019), among whom the rate was 26.9% from 1997-2001 and increased to 34.5% from 2007-2011 in the United States (Interrante et al., 2021). Opioid use during pregnancy is associated with a myriad of adverse outcomes for the mother-child dyad, including higher neonatal intensive care unit (NICU) admission rates and increased hospitalization (Clemans-Cope et al., 2019). From 2010 to 2017, the rate of newborns born with Neonatal Opioid Withdrawal Syndrome (NOWS) has increased by 82% (Hirai et al., 2021). Newborns exposed to *in utero* opioids display characteristics of NOWS, which include decreased body weight, tremors, and high-pitched crying, among others (Weller et al., 2020). In addition, clinical studies have reported that these children score lower in cognitive assessments, display hyperactivity, impulsivity, and aggression (Piccotti et al., 2019; Weller et al., 2020).

The long-term consequences of *in utero* opioid exposure are still largely unknown. Specifically, adolescent and adult abuse risk for commonly used drugs has not been extensively investigated in either the clinical or the preclinical setting. One study found that a higher proportion of individuals that were exposed to drugs of misuse during pregnancy, including heroin, reported misusing alcohol during their lifetime (Nygaard et al., 2020). In addition, one preclinical study reported that adolescent offspring exposed prenatally to methadone have altered sensitivity to alcohol reward and alcohol binge-like drinking, in a sex-dependent manner (Grecco et al., 2022). Other preclinical studies investigating the effects of *in utero* morphine, the active metabolite of heroin, showed that offspring display an enhanced sensitivity to opioids, methamphetamine, and

cocaine. However, the information regarding consumption and preference for other drugs of misuse, including nicotine and alcohol, in offspring exposed to *in utero* morphine, is scarce.

A study utilizing survey data from 1999 to 2020 to investigate the trends in nicotine product use in youths reported that although there has been an overall decline in the use of tobacco/nicotine products, there has been a rise in the popularity of electronic cigarettes (Sun et al., 2021). This is especially relevant as the rate of vaping nicotine has increased in adolescents (Hammond et al., 2021). This makes studying the effects of maternal opioid exposure on nicotine sensitivity in adolescents important. Studies have shown that nicotine affects the adolescent brain and leads to impairments that might be sex-dependent (Mooney-Leber & Gould, 2018). This is especially important considering that the vast majority of women who are on opioid maintenance therapies during pregnancy also use nicotine/tobacco products. Clinical studies have reported that nicotine metabolite ratio (NMR), a marker for nicotine metabolism and clearance, was higher in pregnant women who smoke and were receiving opioid agonist therapies compared to those not receiving therapy, and that the dose of opioid agonist therapy was significantly associated with NMR levels (Kranzler et al., 2021). In addition, preclinical studies found that prenatal and perinatal exposure to opioids alters cholinergic development and activity, specifically during early postnatal days (PNDs) (Robinson, 2000). Even though there is vast evidence for opioid-cholinergic interactions (Drews & Zimmer, 2010), to our knowledge, no study has investigated the effects of *in utero* morphine exposure on nicotine intake and preference during adolescence.

Clinical studies have also reported that nicotine use during adolescence acts as a “gateway drug” to future drug use, including ethanol (Ren & Lotfipour, 2019). Preclinical studies reported that adolescent exposure to nicotine alters adult drug use, which depends on the adolescent period of exposure (Kota et al., 2009), species used (De La Peña et al., 2015; Thomas et al., 2018), and sex (Quijano Cardé et al., 2022; Ruffolo et al., 2021). There is also vast evidence of nicotine-ethanol interactions (Drews & Zimmer, 2010); for example, smoking is a risk factor for alcohol misuse, and use of either drug alters common receptor systems (Lajtha & Sershen, 2010). However, the effects of adolescent nicotine misuse and its subsequent effect on adult ethanol intake in offspring exposed to morphine *in utero* are unknown.

The set of experiments presented in this study unveiled the impact of prenatal-preweaning morphine exposure on adolescent and adult nicotine and/or ethanol intake and preference, and its effect on future drug use risk. We investigated whether offspring exposed to prenatal-preweaning morphine would have higher nicotine intake and preference during adolescence, and consequently consume higher concentrations of ethanol during adulthood, compared to offspring from control dams. The experiments in the present study revealed that prenatal-preweaning morphine alters nicotine preference during adolescence. In addition, ethanol binge-like drinking without prior drug exposure is not altered in offspring from morphine-treated dams; however, nicotine exposure during adolescence increases ethanol binge-like drinking at various concentrations in female offspring from morphine-treated dams. This data further confirms that adolescence is a critical period, and that drug use during this period could have long-lasting consequences, including subsequent drug misuse, on offspring exposed to opioids *in utero*.

4.2. Materials & Methods

4.2.1. Research subjects

C57BL/6J mice were group-housed in standard plastic cages with filter cage tops, corn-cob bedding, and *ad libitum* water and food (Labdiet, 5053, PMI, Brentwood, MO). Animals were housed in a temperature and humidity-controlled 12-hour reverse-light cycle room (lights "off" at 10:00 AM; 65-75^{o}F, 40-60% relative humidity). Female breeders and offspring evaluated during drinking experiments were single-housed at the start of that experiment to ensure accurate measure of drug dose. Shepherd shacks® (Shepherd Specialty Papers, Richland, MI) were placed in the cage as enrichment. For all drinking experiments, bottle placement was alternated (left/right) daily to limit side preference, and bottles in an empty cage were weighed daily to account for fluid leakage. Drinking solutions were presented in 50-mL conical tubes with rubber stoppers and stainless-steel sippers. All experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

4.2.2. Prenatal-Preweaning Morphine Treatment

As the basis for the experiments evaluating offspring outcomes, we utilized an oral saccharin/morphine paradigm for dams that began pre-conception and continued throughout lactation, as described previously (see Chapter 3 methods and preprint by Fleites et al. 2022). Female mice were habituated to one bottle of 0.2% saccharin (Saccharin Sodium Salt Hydrate, Sigma, St. Louis, MO) in filtered water for one week and were then evaluated for baseline physical signs of withdrawal and assigned to either a control-saccharin group or a morphine-treatment group, so that both groups had on

average similar numbers of total signs at the beginning of the experimentation. Signs evaluated include those commonly reported for rodent morphine withdrawal: wet dog shakes, jumping, head shakes, teeth chattering, writhing, and diarrhea (Muldoon et al., 2014). Females assigned to the morphine-treatment group were then given one week of access to one bottle of morphine (Morphine Sulfate, Spectrum Chemical MFG. Corp, New Brunswick, NJ) in saccharin (0.2%) water to promote dependence. The morphine concentration was increased from 0.1 mg/mL free-base (f.b.) morphine (four days) to 0.2 mg/mL f.b. morphine (three days) in saccharin water. At the end of the week of forced exposure, morphine-treated mice were evaluated for spontaneous physical signs during 8-hour withdrawal from 0.2 mg/mL f.b. morphine in saccharin solution. Saccharin-treated (0.2%) female mice were also evaluated for physical signs.

Mice were then transitioned to a continuous two-bottle choice paradigm (C2BC) where they had access to one bottle of filtered water and one bottle of morphine (0.1 mg/mL f.b.) in saccharin (0.2%). Control-saccharin females received one bottle of water and one bottle of saccharin (0.2%) fluid. Daily morphine consumption was reported as morphine intake (mg)/mouse body weight (kg), and preference (%) was calculated as (mLs morphine consumed/mLs total fluid consumed)*100. After one week in the C2BC, mice were again evaluated for spontaneous physical signs after 8-hour withdrawal from morphine. Mice were returned to the C2BC paradigm and physical signs were assessed two days later, after having had continuous access to morphine (morphine-sated state). Control-saccharin females were evaluated for physical signs on the same days as morphine-treated females.

Control-saccharin and morphine-treated females were paired with single-housed C57BL/6J males (~5 hours/day) to mate and were then returned to their own cage to

continue the C2BC paradigm. Females were maintained on the C2BC paradigm throughout gestation and continued until offspring were weaned on PND 21. Offspring of morphine- and saccharin-treated dams were weighed on PND 3, 5, 7, 11, 13, and 15.

4.2.3. Assessment of Ethanol Binge-like Drinking During Adulthood in the Absence of Adolescent Drug Exposure

Since binge drinking is one of the most common patterns of alcohol consumption and can affect individuals across the lifespan (Kanny et al., 2018; Villalonga-Olives et al., 2020), we assessed binge-like ethanol drinking in offspring using a procedure shown to produce clinically relevant, high levels of ethanol drinking (Thiele & Navarro, 2014). Adult offspring (at least 4 months of age) of morphine- and saccharin-treated dams were single-housed for at least one week prior to beginning the drinking-in-the dark paradigm to assess binge-like drinking (Rhodes et al., 2005). Offspring were given one bottle of 20% ethanol (190-proof, Decon Laboratories Inc., King of Prussia, PA) in filtered water (v/v) three hours into the dark cycle. For the first three days, mice had access to the ethanol bottle for two hours, followed by replacement with one bottle of filtered water for the remaining 22 hours. On the fourth day, offspring were given access to the ethanol bottle for only four hours. To assess differences in ethanol intake between the 2-hour and 4-hour DID sessions, we averaged the ethanol intake for the three days of 2-hr drinking. Ethanol intake was calculated as g ethanol consumed/kg mouse body weight.

4.2.4. Adolescent Nicotine Consumption and Preference in the Two-Bottle Choice (2BC) Paradigm

Offspring were assessed during adolescence using a two-part 2BC procedure where we first evaluated their nicotine intake and preference across various concentrations ("nicotine ramp-up"), and then evaluated their motivation to continue drinking nicotine as the solution became progressively bitter ("saccharin ramp-down"). Starting on PND 21, adolescent mice were given two bottles of 2% saccharin in filtered water to habituate them to the drinking solution, to being single-housed, and to being handled. Mice began the nicotine 2BC procedure on PND 28 (+/- 1-2 days) until PND 58. The paradigm features five 2BC sessions with adolescent mice having access to one bottle of nicotine [(-)-Nicotine hydrogen tartrate salt, Glentham Life Sciences, Corsham, United Kingdom] solution and one control bottle. Each session lasted six days, where the mice had access to nicotine in the 2BC for the first four days, and then underwent a 'withdrawal' period for two days. Bottles were weighed every 24 hours, except on the first day of each session, during which they were also weighed 2 hours after the placement of the bottles. The two-hour timepoint models binge-like behavior after two days of the 'withdrawal' period (Thiele, et al 2014).

Nicotine Escalation

As shown in Figure 4-2, the first three sessions of the 2BC procedure will be referred to as the 'nicotine ramp-up' phase, where the concentration of nicotine in 2% saccharin is progressively increased from 50 ug/mL to 200 µg/mL. In session one, mice had access to a 50 ug/mL f.b. nicotine solution in 2% saccharin versus a bottle of 2% saccharin, followed by access to a 100 µg/mL f.b. nicotine solution in 2% saccharin

versus a bottle of 2% saccharin in session two. In session three, they had access to a 200 µg/mL f.b. nicotine solution in 2% saccharin versus a bottle of 2% saccharin.

Saccharin Devaluation

As shown in Figure 4-2, the last two sessions will be referred to as the 'saccharin ramp-down' phase, since the concentration of saccharin was decreased while the concentration of nicotine was kept constant. In the fourth session of the paradigm, mice had access to a 200 µg/mL f.b. nicotine solution in 0.2% saccharin versus a bottle of 0.2% saccharin. Saccharin at 0.2% was used for the first devaluation session since it is the concentration used for our *in utero* dam solutions, and roughly corresponds to a concentration where rodents have peak intake/preference (Sclafani et al., 2010). In the fifth and final session, saccharin was eliminated, and the mice had access to an unsweetened 200 µg/mL f.b. nicotine solution in water versus a bottle of water.

During the two-day 'withdrawal' period, nicotine bottles were replaced with bottles containing the vehicle solution (2% saccharin, 0.2% saccharin, or filtered water) that corresponded to the session that just ended. A separate group of offspring from both dam treatments were used as "saccharin-only" controls, where they were handled identically to the cohort described above, but only received two bottles of the control bottle (no nicotine was ever given). All mice were weighed daily. Nicotine intake (mg/kg) was calculated as mg nicotine consumed/kg mouse body weight. Nicotine preference (%) was calculated as 100*(mLs nicotine consumed/mLs total fluid intake).

4.2.5. Assessment of Binge-like Ethanol Drinking in Adulthood, After Adolescent Drug Exposure

The effect of nicotine/saccharin during adolescence on subsequent ethanol binge-like drinking was assessed in offspring from both dam treatment groups. Beginning at approximately PND 75 (~2.5 weeks after the last nicotine session), offspring were provided with lickometers (built in-house) in the home cage, as previously described (Godynyuk et al., 2019; Quijano Cardé et al., 2022). Briefly, the lickometers use a photo-interrupter beam to passively detect beam breaks reflecting interactions with the sipper, which are used as a proxy for 'drinking' events. This method achieves a higher temporal resolution that permits investigations of drinking microstructure.

Mice were habituated to drinking filtered water from two 15 mL conical bottles fitted with HYDRO-PAC, Inc. valves (HYP-VALVES; Seaford, Delaware) for at least three days. Offspring were then given 4-hour access to two bottles of differing concentrations of ethanol for six sessions. Ethanol bottles were replaced with two bottles of water for the remaining 20 hours at the end of the 4-hour DID session. Ethanol bottles were presented 1 hour after the start of the dark phase of the light cycle. Bottle A contained the 'test' concentration of ethanol which differed across sessions, while Bottle B always contained the 'reference' concentration of ethanol, which was maintained constant at 20% ethanol. During sessions 1-6, mice were given one bottle of 3%, 6%, 10%, 15%, 25%, and 30% ethanol, respectively, versus one bottle of 20% ethanol. On session 7, or the 'testing' session, mice were given 4-hr access to one bottle of filtered water and one bottle of 20% ethanol.

Each session lasted for a minimum of two days to ensure bottle placement was alternated at least once. Additional sessions were included "as-needed" when, e.g., the

device malfunctioned by wires coming loose. Bottles were weighed daily, and ethanol intake was calculated as g ethanol consumed/kg mouse body weight.

4.2.6. Assessment of Taste Perception During Adolescence using the Two-Bottle Choice (2BC) Paradigm

A separate cohort of adolescent mice underwent a taste perception procedure to determine whether prenatal-preweaning opioid exposure affects taste perception. Starting on PND 22, mice underwent three C2BC sessions featuring a bottle of tastant versus a bottle of water. The first session consisted of a bottle of 1% sucrose solution versus one bottle of water. The second session consisted of a bottle of 0.2% saccharin solution versus a bottle of water. The third session consists of a bottle of 0.03 mM quinine solution versus water. Each session lasted two days and the bottle placement was alternated each day to avoid side preference. Taste preference (%) was calculated as (mL tastant consumed/mL total fluid intake)*100.

4.2.7. Statistical Analysis

Data are expressed as mean +/- standard error of the mean and were analyzed using Graphpad PRISM 9. A p-value of <0.05 was considered statistically significant. ROUT test (Q=1%) in Prism was used to remove significant outliers. For the adolescent nicotine data, the ‘nicotine ramp up’ phase (sessions 1-3) and the ‘saccharin ramp-down’ phase (sessions 3-5) were analyzed separately. A repeated-measures (RM) three-way ANOVA was first used to evaluate the potential effect of sex, session, and dam treatment for adolescent nicotine data. When there was no effect of sex, males and

females were combined for subsequent analysis in a RM two-way ANOVA. Adolescent taste perception and adult ethanol drinking (Figure 4-1) data was analyzed using a two-way ANOVA. For adult ethanol binge-like drinking data after adolescent drug exposure, analysis revealed a main effect of sex, so female and male drinking data were analyzed separately, and shown separately in graphical form. Data for adult ethanol drinking was analyzed using a RM three-way ANOVA to evaluate an effect/interaction of ethanol concentration, dam treatment, and adolescent treatment for each sex. A mixed-effects model was used for any data sets that were missing values at a specific timepoint (ex. bottle pushed out, etc). Where appropriate, a Sidak test was used for post hoc analysis.

Lickometer data were analyzed using a custom-written VBA macro in Microsoft Excel. Events lasting more than 15 seconds were excluded, as these were considered likely to be spurious. We assessed various parameters, including latency to approach (i.e. the first interaction with the bottle), number of bouts, and number of bouts during the first 30 minutes after the first bout occurred. Bouts were defined as events with cumulative duration of ≥250 ms and inter-event interval of <1000 ms.

4.3. Results

4.3.1. Adult ethanol binge-drinking is not altered in offspring from morphine-treated dams

Ethanol binge drinking affects over 7% of the population and can have deleterious effects, including increased risk for subsequent alcohol use and premature death (Waszkiewicz et al., 2018). We utilized the classic drinking-in-the-dark (DID) paradigm where offspring had limited access to one bottle of ethanol to assess the effects of *in utero* morphine exposure on ethanol binge-like drinking, which has not been

previously evaluated. Although females drank significantly more than males, as previously reported (Thiele & Navarro, 2014), we did not find a main effect of dam treatment on ethanol binge-like consumption for either sex (Figure 4-1). There was a significant main effect of DID session for both males (RM Two-Way ANOVA; $F(1, 24) = 86.43$, $p < 0.0001$; Figure 4-1A) and females (RM Two-Way ANOVA; $F(1, 24) = 76.62$, $p < 0.0001$; Figure 4-1B), where both sexes drank more on the 4-hour session compared to the 2-hour session.

These data suggest that adult ethanol binge-like drinking is not altered in male and female offspring exposed to prenatal-preweaning morphine.

4.3.2. Adolescent 2-hour nicotine preference is altered in offspring from morphine-treated dams

We were interested in assessing nicotine intake and preference during adolescence in offspring from morphine-treated dams, given the cross-tolerance between nicotine and morphine (Drews & Zimmer, 2010). We used a 5-session 2BC paradigm where we assessed sensitivity to increasing nicotine concentrations in the first three sessions ("nicotine ramp up"), while in the last two sessions we assessed the offspring's motivation to maintain the nicotine dose they were previously drinking as the solution became increasingly bitter due to decreasing saccharin content ("saccharin ramp down").

There was no significant effect of sex on nicotine intake during adolescence, which was consistent with one study (McClellan Stine et al., 2003), but not with others (Bagdas et al., 2020; Klein et al., 2004; Lee et al., 2017). When we assessed 2-hour

nicotine intake after a 2-day abstinence period (Figure 4-2A), we did not find a significant main effect or interaction of dam treatment during the 'nicotine ramp-up' phase. When we assessed 2-hour nicotine intake during the 'saccharin ramp-down' phase, we found a significant interaction between dam treatment and session (RM mixed effects analysis; $F(2, 78) = 3.545$, $p = 0.0336$), where numerically offspring from morphine-treated dams drank less nicotine during session 4, although post-hoc analysis did not detect any statistical significance (p=0.1118).

When we analyzed 2-hour nicotine preference, we found a significant interaction (p=0.0237) between dam treatment, session, and sex during the 'saccharin ramp-down' phase, so male and female offspring were analyzed separately. We did not observe a significant main effect or interaction of dam treatment for 2-hour preference during the 'nicotine ramp-up' for male (Figure 4-2B) and female (Figure 4-2C) offspring. As shown in Figure 4-2C, we also did not find a significant main effect or interaction of dam treatment for female offspring's 2-hour nicotine preference during the 'saccharin ramp-down' phase. However, for male offspring, we did observe a significant interaction of dam treatment and session (RM mixed effects analysis; $F(2, 40) = 6.475$, $p = 0.0037$; Figure 4-2B) during the 'saccharin ramp-down' phase. Post-hoc tests revealed a trend (p=0.0510) for male offspring from morphine-treated dams to have lower 2-hour nicotine preference than male control offspring during session 4 when saccharin was decreased to 0.2%.

Given the subtle changes in 2-hour nicotine intake and preference, we examined whether those changes persisted, affecting daily intake and preference. Our analyses indicated that the effect observed for preference at the 2-hour timepoint was transient, as we did not find a significant main effect or interaction of dam treatment for 24-hour

nicotine intake (Figure 4-2D) and preference (Figure 4-2E). In addition, we did not detect a main effect of dam treatment between offspring for 2-hour (Supplemental Figure 4-1A) and 24-hour (Supplemental Figure 4-1B) total fluid intake.

This suggests that there are transient differences in nicotine intake and preference among offspring from both dam treatments.

4.3.3. Adolescent taste perception is altered in offspring

We were interested in determining whether the difference in 2-hour nicotine preference between offspring from control dams and morphine-treated dams was due to changes in adolescent taste perception. We hypothesized that the decrease in 2-hour nicotine preference when the saccharin concentration was reduced from 2% to 0.2% was due to lower baseline "sweet" preference in offspring from morphine-treated dams. We analyzed adolescent offspring's preference and intake for three tastants: (1) sucrose: sweet and caloric, (2) saccharin: sweet and non-caloric, and (3) quinine: bitter, which might model the bitterness of nicotine. When we assessed 1% sucrose preference, there was no main effect of dam treatment. Preliminary results suggest a significant interaction between dam treatment and sex (Two-Way ANOVA; $F(1, 24) = 4.878$, $p = 0.0370$; Supplemental Figure 4-2A) for 1% sucrose preference, but no significant difference for sucrose intake (Supplemental Figure 4-2D). In addition, when we assessed the preference for 0.2% saccharin, we found a significant main effect of dam treatment (Two-Way ANOVA; $F(1, 22) = 5.878$, $p = 0.0240$; Supplemental Figure 4-2B) and sex (Two-Way ANOVA; $F(1, 22) = 11.48$, $p = 0.0026$; Supplemental Figure 4-2B), but no significant interaction. There was a trend ($p=0.0515$) for female control

offspring to have higher 0.2% saccharin preference than male control offspring. In addition, there was a main effect of sex for 0.2% saccharin intake (Two-Way ANOVA; $F(1, 25) = 7.797$, $p = 0.0099$; Supplemental Figure 4-2E), but not dam treatment. Finally, we found no significant difference for 0.03 mM quinine preference (Supplemental Figure 4-2C) or intake (Supplemental Figure 4-2F).

Together these results suggest that prenatal-preweaning morphine exposure alters adolescent 2-hour nicotine preference when the saccharin is decreased to 0.2%, which could possibly be due to changes in adolescent perception of sweet taste.

4.3.4. Female offspring from morphine-treated dams that were exposed to nicotine during adolescence have increased adult ethanol binge-like drinking

As shown in Figure 4-1, we established that ethanol binge-like drinking did not differ between offspring from morphine-treated dams and control offspring during adulthood. Considering that adolescent exposure to drugs of abuse, including tobacco/nicotine, can lead to subsequent ethanol misuse (Ren & Lotfipour, 2019), we compared adult ethanol binge-like drinking in offspring exposed to either nicotine or saccharin during adolescence. We hypothesized that nicotine exposure during adolescence would lead to greater ethanol intake during adulthood in offspring from morphine-treated dams. To assess binge-like drinking, offspring had limited access (4-hr) to ethanol in a 2BC where one ethanol bottle was the ‘test’ concentration that increased across sessions, and the second bottle contained a ‘reference’ concentration (20% ethanol) that stayed the same across the sessions. We were interested in

assessing ethanol intake between two ethanol concentrations during each session to investigate the sensitivity to various ethanol concentrations, obtain an ethanol dose-response curve, and obtain measurements related to drinking microstructure via the use of lickometers.

To model the “open bar” studies performed in human subjects (Zimmermann et al. 2013) we assessed whether offspring were more likely to drink a low ethanol concentration solution (similar to a beer) or a higher ethanol concentration (similar to a liquor shot). We used the average ethanol intake for the test concentration (3-30%) to report an ethanol concentration response curve (Figure 4-3). As shown in Figure 4-3, ethanol intake from the test bottle changed significantly across concentrations in all groups, where offspring from each adolescent treatment increased their ethanol intake from the first session (3%) to the last one (30%). There was no significant main effect or interaction of dam treatment for male offspring exposed to nicotine or saccharin during adolescence (Figure 4-3A), suggesting that maternal morphine exposure does not shift the concentration-response curve for ethanol in these offspring. Interestingly, we observed a significant interaction between dam treatment and adolescent treatment in female offspring (RM three-way ANOVA; $F(1,31) = 4.927$, $p = 0.0339$; Figure 4-3B). Because of this, we further analyzed female control and morphine-treated offspring exposed to the same adolescent treatment and found a significant main effect of dam treatment (RM two-way ANOVA; $F(1,17) = 5.826$, $p = 0.0274$; Figure 4-3B) between control and morphine-treated female offspring that were exposed to nicotine during adolescence, but not between those exposed to saccharin. This suggests that nicotine exposure during adolescence leads female offspring from morphine-treated dams to

drink significantly more from the test bottle compared to female offspring from control dams.

Since female offspring from morphine-treated dams that were exposed to nicotine during adolescence displayed increased ethanol intake at various test concentrations, we hypothesized that they would also have higher total ethanol intake when considering the dose consumed from both ethanol bottles in each session. Surprisingly, when we assessed average total ethanol intake in each session (sum of ethanol intake from test concentration bottle and reference concentration bottle), we did not see a significant main effect or interaction of dam treatment for male or female offspring exposed to nicotine or saccharin during adolescence (Supplemental Figure 4-3). In addition, since offspring were exposed to ethanol for 6 sessions, we expected all adolescent treatment groups to maintain their preferred ethanol dosage by drinking similar levels of ethanol during the last testing session (7), when they had a choice between water and 20% ethanol. However, as shown in Supplemental Figure 4-3, all adolescent treatment groups decreased their ethanol intake on session 7 when compared to their session 6 intake, suggesting that prior limited-access exposure to ethanol was not sufficient to drive offspring to drink similar levels of ethanol when they had a choice between water and ethanol.

Similarly, when we assessed ethanol preference across all the sessions, we also expected for offspring from morphine-treated dams to have higher ethanol preference and for all offspring to prefer the ethanol bottle during the testing session (session 7). However, as shown in Figure 4-4, we found no significant main effect of dam treatment or interaction between dam treatment and adolescent treatment for male and female offspring. This again suggests that maternal morphine exposure does not alter

offspring's preference for ethanol, even if offspring were exposed to nicotine or saccharin during adolescence.

Together, these data suggest that female offspring from morphine-treated dams that were exposed to nicotine during adolescence increase their ethanol intake at various concentrations during adulthood, although there is no change in ethanol preference or total session ethanol intake.

In addition to the gross measure of ethanol intake and preference, we were also interested in assessing drinking topography in our offspring. Both clinical and preclinical studies have reported on changes in drinking microstructure as being important for binge drinking and AUD severity, including rate of consumption, frequency and duration of heavy drinking, latency to first drink, and number of drinking bouts (Azarov & Woodward, 2014; Baker et al., 2017; Carpenter et al., 2019; Leeman et al., 2018; Read et al., 2008). We further investigated whether changes in drinking topography, reflected by parameters related to bout, could explain the increase in ethanol intake at various ethanol concentrations in female offspring exposed to nicotine during adolescence after prenatal-preweaning morphine exposure. We did not find a significant main effect of dam treatment for total number of bouts, total bout duration, average bout duration during each 4-hour session, and latency to first bout (data not shown) in any of our treatment groups.

Previous work by Baker *et al.* (2017) reported different drinking patterns among primates that were very heavy drinkers (VHD) and ones that were low drinkers (LD), where VHD drank in a "gulping style" (reduced drinking time but with same volume) and LD drank in a "sipping style" (increased latency to first drink and many bouts of small

volume), which corresponded with levels of intoxication. We were therefore interested in understanding whether offspring consumed high levels of ethanol over shorter periods of time and measured the number of bouts that occur 30 minutes after the first bout for the test concentration bottle as a way of characterizing the frequency of consumption once the initial bout occurs. We did not observe a significant main effect of dam treatment in male offspring exposed to nicotine or saccharin during adolescence (Figure 4-5A). Instead, we found a significant main effect of ethanol, suggesting that bout number after the first initial bout changes across ethanol concentrations (Figure 4-5).

We also investigated the average time it took for offspring to approach the test concentration bottle, as reduced latency to first drink and time to bout initiation have been associated with binge drinking and increased motivation/urge to drink (Darevsky et al., 2019; Davidson et al., 1996; Rose et al., 2010). We observed no significant main effect or interaction of dam treatment on latency to interact with the test concentration bottle for male or female offspring. However, we did find a main effect of ethanol concentration, which suggests that as the concentration increases, the latency to approach the test bottle increases, similar to another study (Eastwood et al., 2014), possibly reflecting either the aversiveness of that concentration or increased sensitivity to ethanol.

Taken together, these data suggest that prenatal-preweaning morphine exposure followed by exposure to nicotine during adolescence led to higher ethanol consumption at various test concentrations in adult female offspring. These effects were not seen in

female offspring exposed to saccharin during adolescence, or in male offspring exposed to nicotine or saccharin during adolescence.

4.4. Discussion

The experiments presented in this study demonstrate sex-specific alterations in the trajectory of drug intake among offspring exposed to prenatal-preweaning morphine. In adolescents, prenatal-preweaning morphine altered the preference to nicotine as a function of the amount of saccharin present in the drinking solution, possibly reflecting changes in taste perception. In adulthood, although prenatal-preweaning morphine exposure alone did not alter ethanol binge-like drinking, female offspring from morphine-treated dams displayed increased binge-like drinking behavior after exposure to nicotine during adolescence. Therefore, maternal opioid exposure and nicotine exposure during adolescence alters alcohol drinking in adult offspring in a sex-specific manner.

The results of the nicotine 2BC experiment during adolescence indicate that there are changes in nicotine preference during the 2-hour timepoint of session four, among offspring from control and morphine-treated dams. Specifically, male offspring from morphine-treated dams had lower 2-hour nicotine preference when the saccharin decreases (2% vs 0.2%). There are multiple explanations for this finding. First, we could conclude that developmental exposure to a sweetened morphine solution results in the sweet taste of saccharin contributing less to the reinforcing and/or rewarding properties of nicotine than in control mice. Alternatively, one might conclude that control offspring increase their nicotine intake and preference during the 2-hour timepoint of this session because the bitterness of the nicotine/saccharin solution is not as aversive to them as it

is to the offspring from morphine-treated dams. Indeed, one study reported concentration-response curves for saccharin and showed that 2% saccharin is more aversive than lower concentrations of saccharin, like 0.2% (Domjan & Gillan, 1976). This is possibly due to a metallic and bitter "off-taste" that is reported from saccharin consumption at high concentrations (Glendinning, 2018). It is also possible that the decrease in intake for the 0.2% saccharin-nicotine containing solution in morphine-treated offspring may be due to prenatal-preweaning pairing of morphine with 0.2% saccharin, as seen in a study that paired heroin with saccharin and saw a decrease in subsequent saccharin-only intake (Grigson et al., 2000). It is important to note that the difference observed at the 2-hour time point disappears when looking at the 24-hour preference of session 4, suggesting that the phenomenon does not affect the daily amount of nicotine exposure. It is also interesting to note that the difference is not significant in session 5, which marks the second devaluation session. Finally, the increase in nicotine intake/preference in control offspring might result from *in utero* exposure to 0.2% saccharin solution. Saccharin and other artificial sweeteners can reach the offspring through the placenta and the breast milk, and can have lasting effects, including changes in sweet taste perception (Cohen-Addad et al., 1986; Goran et al., 2019; Sylvetsky et al., 2015; Zhang et al., 2011). Interestingly, our preliminary data suggests that female offspring from control dams might have a higher saccharin preference than male offspring. Our data also suggest that adolescent females overall have higher saccharin intake, which is in-line with both clinical and preclinical data (Carroll et al., 2008; Kamens et al., 2018). Higher preference for sweet tastants has been associated with increased drug self-administration and intake (Blednov et al., 2008; Carroll et al., 2002; Dess et al., 1998). Therefore, additional work is needed to determine

whether the change in adolescent taste perception is robust and continues until adulthood.

There is evidence from both clinical and preclinical data that exposure to tobacco/nicotine during adolescence can increase the risk for future drug use. We, however, did not detect an increase in adult ethanol drinking after adolescent nicotine exposure in our control offspring. One possible explanation for this result could be that the control offspring had lower nicotine intake (~1-5 mg/kg/day) than what is needed to observe a robust effect on adult nicotine intake, possibly do to the fact that nicotine was administered in a 2BC and offspring had lower than a 50% preference for the nicotine-containing solution. Indeed, our lab reported that female C57BL/6J mice, but not males, display increased ethanol intake in adulthood after adolescent exposure to nicotine (Quijano Cardé et al., 2022). However, the mice in that study were drinking under a forced nicotine access paradigm and thus were consuming an average of ~30-50 mg/kg/day (Quijano Cardé et al., 2022). Interestingly, even though nicotine intake was not different between female offspring from control and morphine-treated dams, the interaction between opioids *in utero* and adolescent nicotine was sufficient to increase ethanol binge-like drinking in adulthood. This opioid-nicotine interaction is of interest considering that this effect was not seen in female offspring from morphine-treated dams that were exposed to saccharin during adolescence (Figure 4-3), and in offspring that received no adolescent exposure (Figure 4-1).

Our data indicate that adult females born to morphine-treated dams have altered sensitivity to various concentrations of ethanol if they were exposed to nicotine during adolescence. We tested a series of alcohol concentrations to evaluate ethanol sensitivity by measuring intake and preference. We found that female offspring exposed to nicotine

do not seem to decrease their ethanol intake and preference like the other offspring when presented with a 30% ethanol-containing solution. This could signify that adolescent nicotine exposure, specifically in female offspring, reduces sensitivity to the aversive elements of high concentrations of ethanol. Alternatively, female offspring exposed to morphine/nicotine could have impaired cognitive flexibility, which has been reported in humans and animal models exposed to drugs of abuse (Nesic et al., 2011; Ortega et al., 2013; Stalnaker et al., 2009). In our study, mice first learned that the reference bottle was always higher in concentration (20%) than the test bottle (3-15%) during sessions 1-4, but then, possibly due to cognitive inflexibility, may not have learned to drink from the reference bottle during sessions 5 and 6 when it was actually a lower concentration (i.e. when ethanol concentration in the test bottle increased to 25% and 30%). Further work is needed to elucidate the mechanism by which adolescent nicotine exposure increases binge ethanol intake in female offspring exposed to prenatal-preweaning morphine.

We used lickometers to further evaluate drinking microstructure. Similar to Grecco *et al*. (2022), who investigated the effects of prenatal methadone exposure on ethanol drinking microstructure using a limited access paradigm, we did not find that maternal morphine exposure significantly altered drinking microstructure. For example, the authors did not find significant differences in beam breaks, total sum of beam breaks, and duration of beam breaks between offspring from both dam treatments (Grecco et al., 2022). They found that increasing the quinine concentration in their aversion-resistant drinking experiment increased the latency to drink, which was similar to our results where the latency to approach increased as the concentration of ethanol increased. Other studies have reported changes in “front-loading” behavior, which is where rodents

drink the majority of their ethanol in the beginning of the drinking session (Darevsky et al., 2019; Linsenbardt & Boehm, 2014; Quijano Cardé et al., 2022; Wilcox et al., 2014). However, we did not find significant differences in the number of interactions and bouts during the first 30 minutes of each session for the various ethanol test concentrations (data not shown). Although there was no significant difference in drinking microstructure in offspring from morphine-treated dams, this might be in line with the notion that offspring exposed to opioids throughout gestation are thought to behave overall "normal" with slight differences in behavior. For example, clinical data report overall changes in behavioral and cognitive assessments in children exposed to *in utero* opioids, even though their scores still remain within normal range (Nygaard et al., 2015; Skumlien et al., 2020). Whether these children's behavioral phenotype changes after "multiple hits", like nicotine exposure during adolescence, is still largely unknown, but this could have implications for adolescent and adult drug use. The concept that a "first hit" (i.e. *in utero* opioid exposure) can push individuals to a threshold to develop some subtle changes in behavior, and then a "second/multiple hit" is what pushes individuals to develop severe phenotypes, is a model that has gained traction in both clinical and preclinical research (Davis et al., 2016; Girirajan & Eichler, 2010; Klug et al., 2015; Tsukamoto et al., 2009), and is an important consideration moving forward, which the present study has highlighted.

The changes described in this study could arise from alterations in the endogenous opioid system after prenatal-preweaning morphine exposure. Previous studies have shown that *in utero* morphine exposure alters mu opioid receptor (MOR) densities in various brain regions, including the striatum and hippocampus, which are important for drug-taking and reward phenotypes (Chiou et al., 2003; Schindler et al.,

2004; Šlamberová et al., 2003; Vathy et al., 2003). Chiou *et al.* (2003) showed that offspring had decreased MOR density in the striatum after exposure to a maternal morphine paradigm that started pre-conception and extended throughout lactation, similar to the paradigm we use for the dyads. Also, MOR densities are differentially altered in the hippocampus of female offspring exposed to prenatal morphine and this is mediated by reproductive hormones, where decreased MOR density is observed in ovariectomized females, but treatment with estrogen and progesterone increases MOR density (Šlamberová et al., 2003). Such phenomenon could explain why the effect on ethanol intake is specific to females if opioid receptor and/or peptide levels were indeed decreased in those offspring. Thus, alterations in the opioid receptor system by prenatal-preweaning morphine exposure could underlie the complex interactions among opioids, nicotine, and alcohol in a sex-dependent fashion. In addition, future work in offspring from morphine-treated dams should investigate the effects of adolescent ethanol exposure on subsequent nicotine use risk, as the prevalence of binge drinking in young adults is high and might be the more common drug to be encountered first (Krieger et al., 2018).

In summary, the present study revealed that prenatal-preweaning opioid use combined with adolescent drug exposure may be a risk factor for future drug use in vulnerable populations of offspring exposed to *in utero* opioids. Epidemiological and longitudinal studies are needed to investigate if this finding is clinically relevant, and if so, what are the mechanisms behind this phenomenon.

4.5. Figures

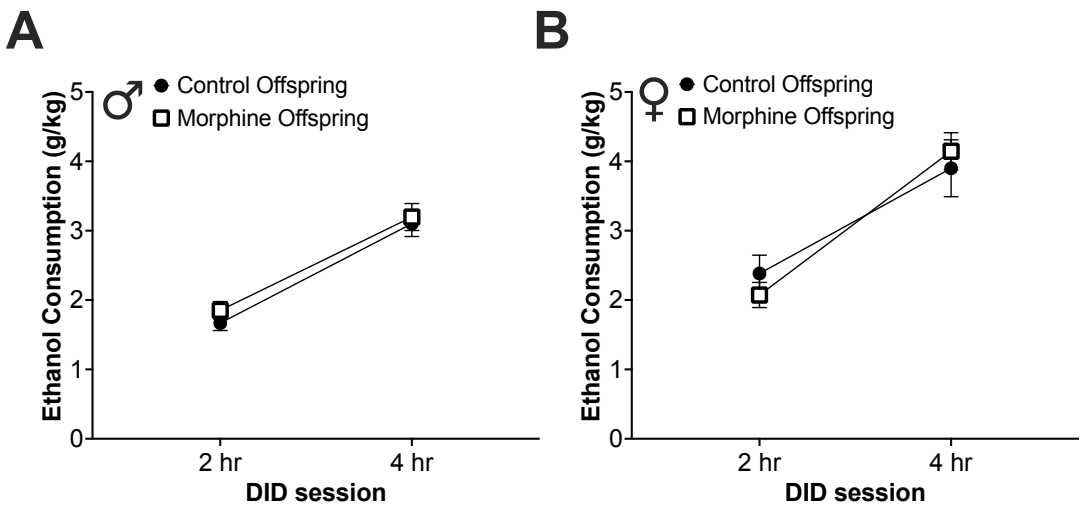


Figure 4-1: No change in ethanol binge-like drinking in adult offspring from morphine-treated dams.

(**A & B**) Ethanol intake (g/kg) was accessed using the limited access (2- or 4-hr) to one bottle of 20% ethanol paradigm in male (n=14,12) **(A)** and female (n=13,13) **(B)** offspring from control and morphine-treated dams.

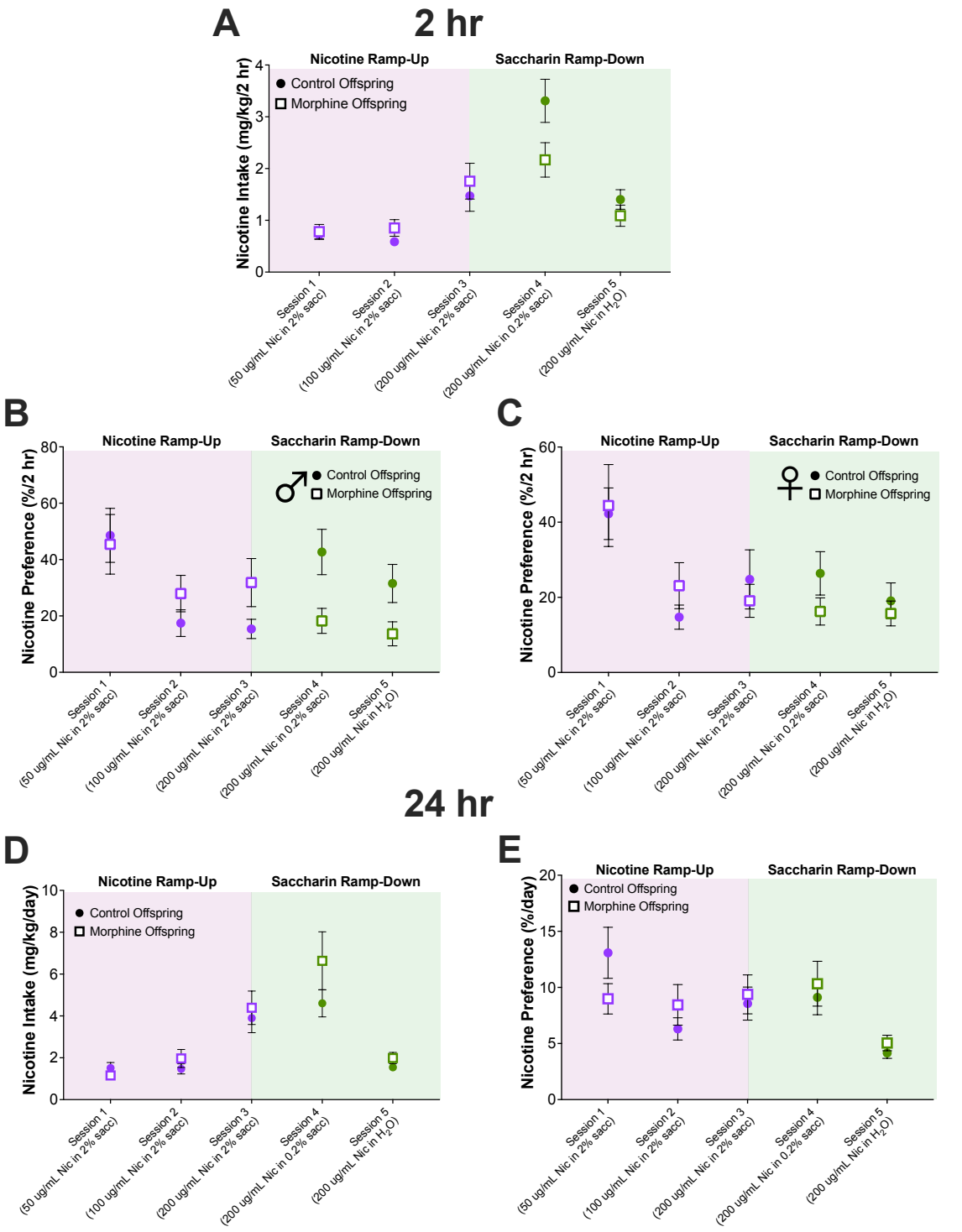


Figure 4-2: Investigation of adolescent 2-hr and 24-hr nicotine intake and preference in a two-bottle choice (2BC) paradigm in offspring.

(**A**) Nicotine intake during the first two hours of the first day of the session during 'nicotine ramp-up' (sessions 1-3) and 'saccharin ramp-down' (sessions 3-5) between offspring exposed from morphine-treated dams (n=22) and control offspring (n=21). Main effect of session for nicotine ramp-up (RM mixed effects model; $F(1.285, 52.06) = 15.43$, $p < 0.0001$) and saccharin ramp-down (RM mixed effects model; $F(1.699, 66.24) = 16.43$, $p < 0.0001$). (**B & C**) Nicotine preference shown for male (**B**) (n=11,11) and female (**C**) (n=10,11) offspring from control and morphine-treated dams during the first two hours of the session during 'nicotine ramp-up' and 'saccharin ramp-down'. (**B**) Main effect of session for 'nicotine ramp-up' (RM mixed effects model; $F(1.809, 35.27) = 7.762$, $p = 0.0022$). (**C**) Main effect of session for 'nicotine ramp-up' (RM mixed effects model; $F(1.830, 33.86) = 9.871$, $p = 0.0006$). (**D & E**) Nicotine 24-hour intake (**D**) and 24-hour preference (**E**) during 'nicotine ramp-up' (sessions 1-3) and 'saccharin ramp-down' (sessions 3-5) between offspring exposed from morphine-treated dams (n=22) and control offspring (n=21). (**D**) Main effect of session for 'nicotine ramp-up' (RM mixed effects model; $F(1.251, 46.29) = 39.06$, $p < 0.0001$) and 'saccharin ramp-down' (RM mixed effects model; $F(1.676, 63.71) = 17.43$, $p < 0.0001$). (**E**) Main effect of session for 'nicotine ramp-up' (RM mixed effects model; $F(1.412, 52.26) = 7.107$, $p = 0.0049$) and 'saccharin ramp-down' (RM mixed effects model; $F(1.919, 73.90) = 11.41$, $p < 0.0001$).

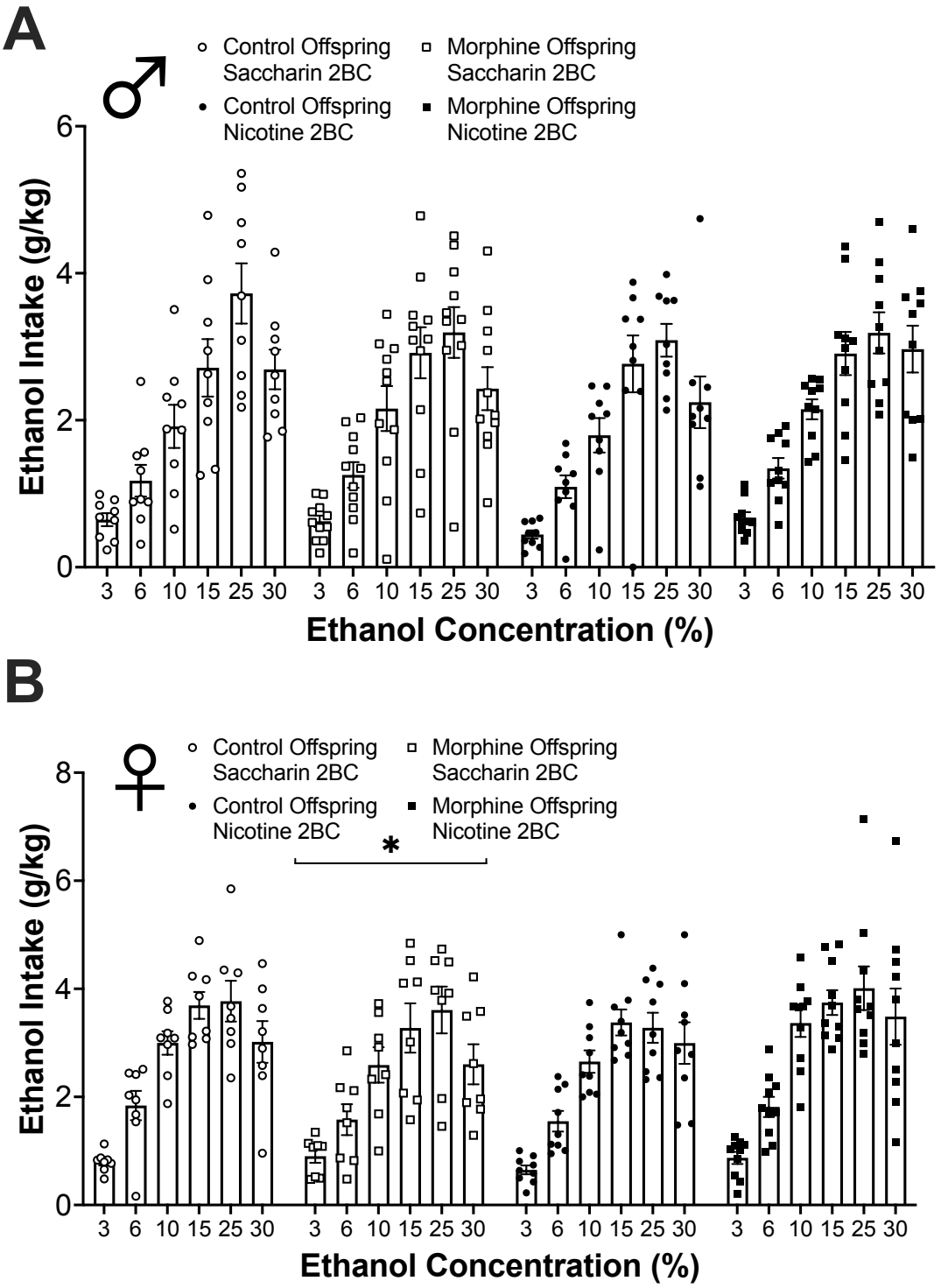

A
♂
Control Offspring Saccharin 2BC
Morphine Offspring Saccharin 2BC
Control Offspring Nicotine 2BC
Morphine Offspring Nicotine 2BC
Ethanol Intake (g/kg)
0
2
4
6
3 6 10 15 25 30 3 6 10 15 25 30 3 6 10 15 25 30 3 6 10 15 25 30
Ethanol Concentration (%)
B
♀
Control Offspring Saccharin 2BC
Morphine Offspring Saccharin 2BC
Control Offspring Nicotine 2BC
Morphine Offspring Nicotine 2BC
*
Ethanol Intake (g/kg)
0
2
4
6
8
3 6 10 15 25 30 3 6 10 15 25 30 3 6 10 15 25 30 3 6 10 15 25 30
Ethanol Concentration (%)

Figure 4-3: Investigation of adult ethanol binge-like drinking in a limited access (4-hr) two-bottle choice (2BC) paradigm in offspring exposed to nicotine or saccharin 2BC during adolescence.

(**A & B**) Average ethanol intake for the test concentration (3-30% ethanol) bottle across sessions in male (n=9-11) (**A**) and female (n=8-10) (**B**) offspring from control or morphine-treated dams that were exposed to nicotine or saccharin 2BC during adolescence. (**A**) Main effect of concentration (RM three-way ANOVA; $F(2.528, 88.47) = 77.95$, $p < 0.0001$). (**B**) Main effect of concentration (RM three-way ANOVA; $F(2.305, 71.44) = 64.16$, $p < 0.0001$).

* $p<0.05$, main effect of dam treatment (comparing control & morphine-treated female offspring exposed to nicotine during adolescence)

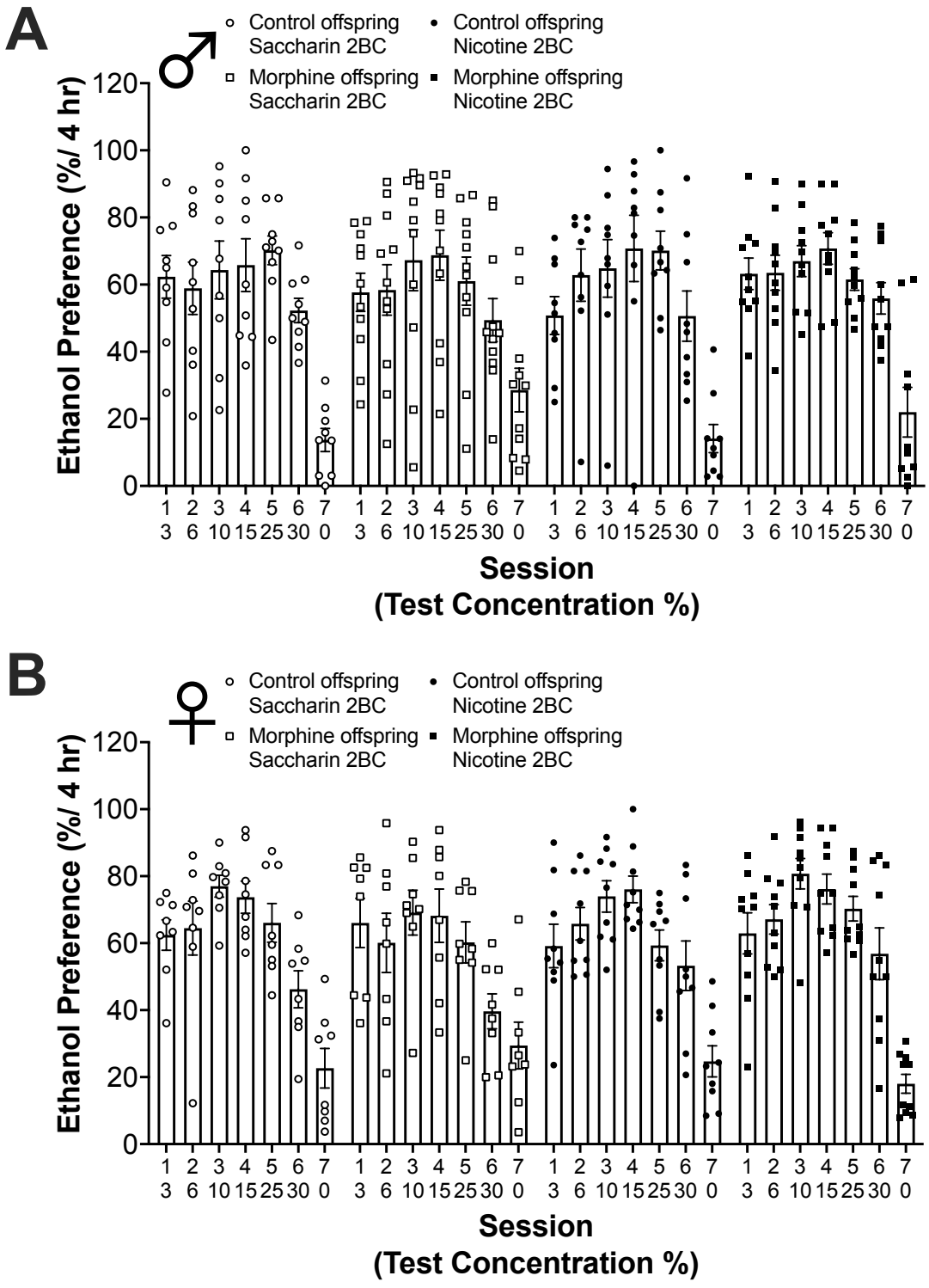


Figure 4-4: No difference in ethanol preference in adult offspring exposed to nicotine or saccharin 2BC during adolescence.

We investigated ethanol binge-like drinking in a limited access (4-hr) two-bottle choice (2BC) paradigm in offspring exposed to nicotine or saccharin 2BC during adolescence. (**A & B**) Average ethanol preference for the test concentration bottle across sessions in male (n=9-11) (**A**) and female (n=8-10) (**B**) offspring from control or morphine-treated dams that were exposed to nicotine or saccharin 2BC during adolescence. (**A**) Main effect of concentration (RM three-way ANOVA; $F(2.653, 92.85) = 37.14$, $p < 0.0001$). (**B**) Main effect of concentration (RM three-way ANOVA; $F(3.380, 104.8) = 43.06$, $p < 0.0001$).

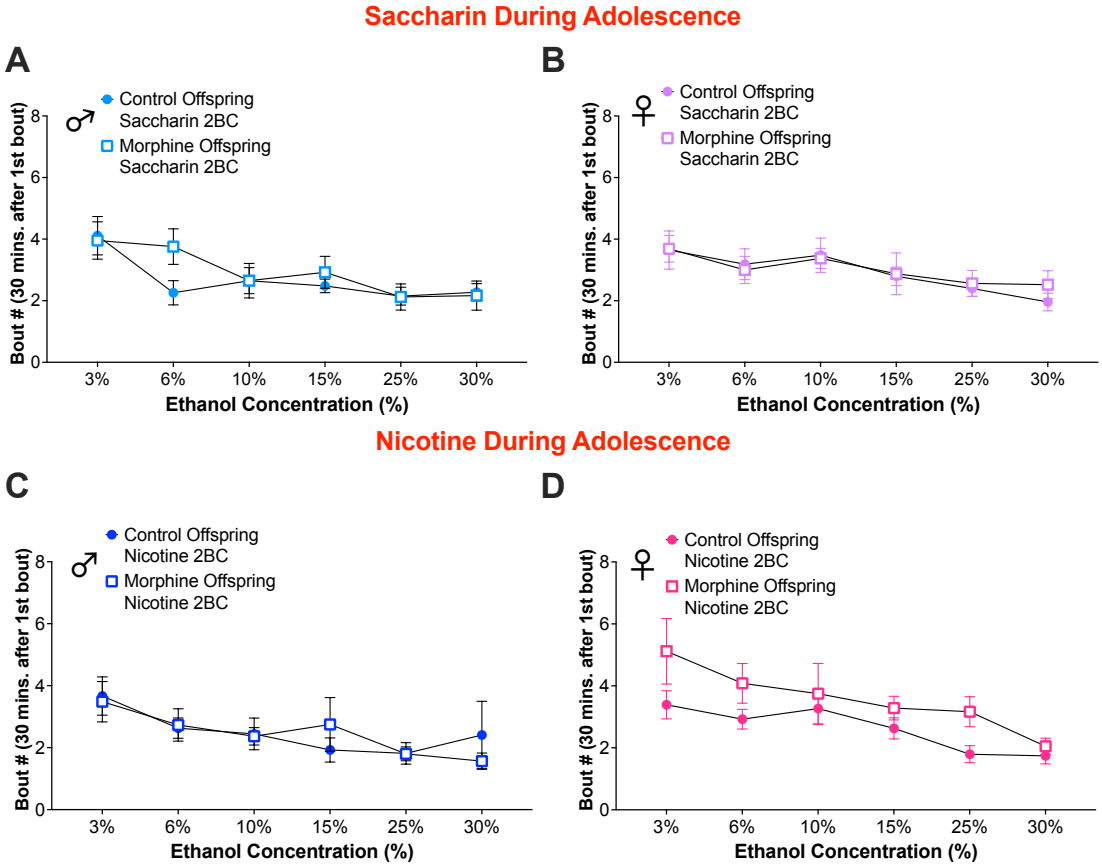
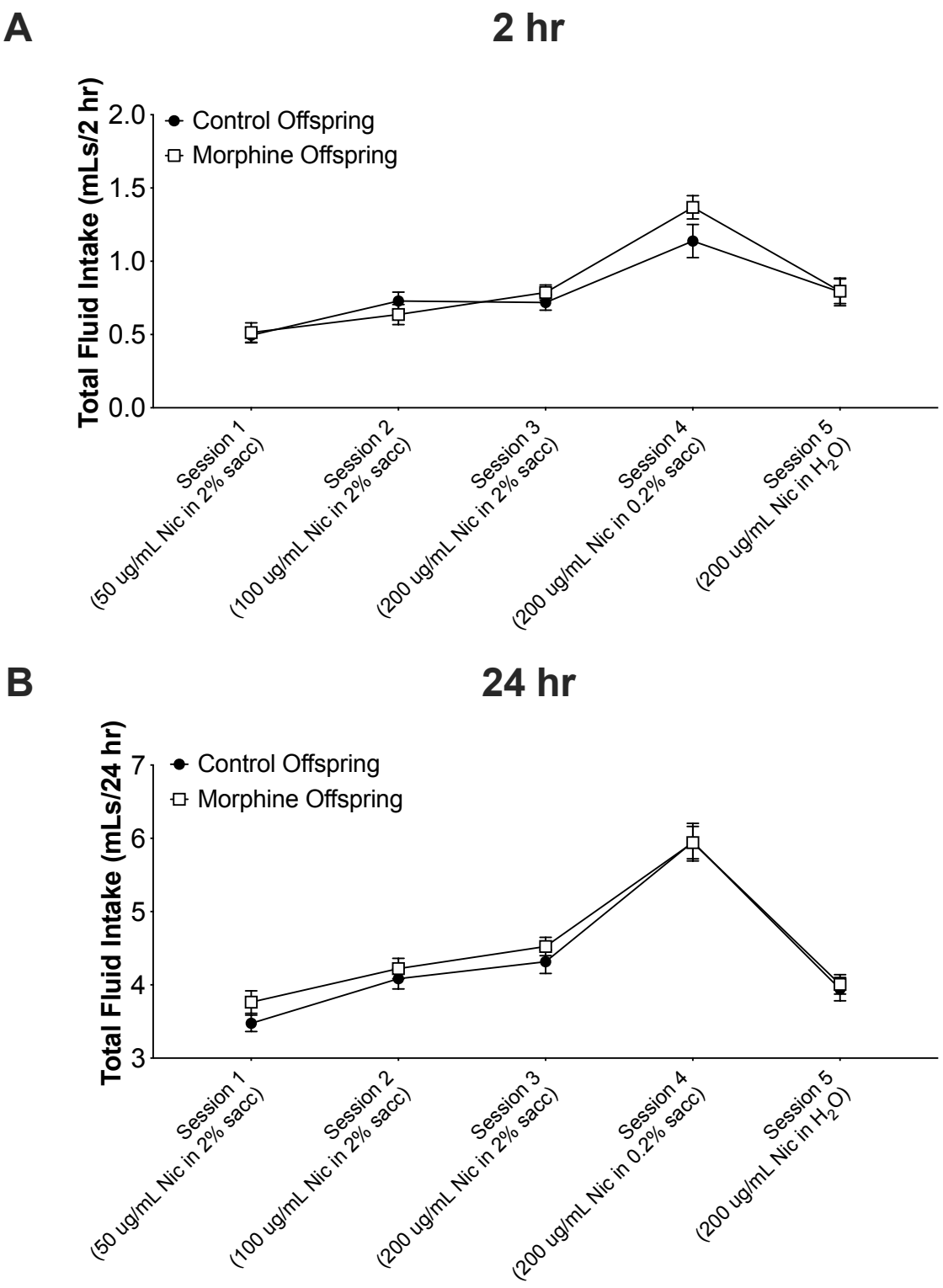


Figure 4-5: No difference in the number of bouts that occurred 30 minutes after the first bout in adult offspring exposed to saccharin or nicotine 2BC during adolescence, measured using lickometers.

We investigated ethanol binge-like drinking microstructure using lickometers in a limited access (4-hr) two-bottle choice (2BC) paradigm in offspring exposed to saccharin or nicotine 2BC during adolescence. (**A & B**) Average number of bouts that occurred 30 minutes after the first bout for the test concentration (3-30% ethanol) bottle across sessions in male (n=9,11) (**A**) and female (n=8,8) (**B**) offspring from control or morphine-treated dams that were exposed to saccharin 2BC during adolescence. (**C & D**) Average number of bouts that occurred 30 minutes after the first bout for the test concentration (3-30% ethanol) bottle across sessions in male (n=9,10) (**C**) and female (n=9,10) (**D**) offspring from control or morphine-treated dams that were exposed to nicotine 2BC during adolescence.

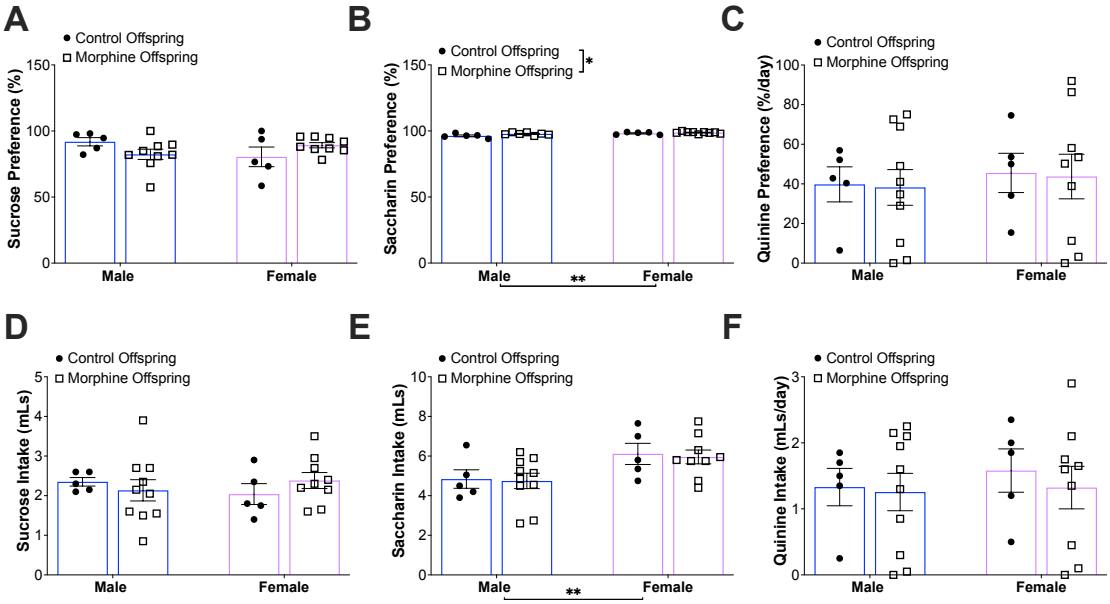
Main effect of concentration for male (RM three-way ANOVA; $F(4.014, 140.5) = 6.589$, $p < 0.0001$) and female offspring (RM three-way ANOVA; $F(3.379, 104.7) = 8.313$, $p < 0.0001$).

4.6. Supplemental Figures


A
2 hr
Total Fluid Intake (mLs/2 hr)
2.0
1.5
1.0
0.5
0.0
Control Offspring
Morphine Offspring
Session 1 (50 ug/mL Nic in 2% sacc)
Session 2 (100 ug/mL Nic in 2% sacc)
Session 3 (200 ug/mL Nic in 2% sacc)
Session 4 (200 ug/mL Nic in 0.2% sacc)
Session 5 (200 ug/mL Nic in H_2O)
B
24 hr
Total Fluid Intake (mLs/24 hr)
7
6
5
4
3
Control Offspring
Morphine Offspring
Session 1 (50 ug/mL Nic in 2% sacc)
Session 2 (100 ug/mL Nic in 2% sacc)
Session 3 (200 ug/mL Nic in 2% sacc)
Session 4 (200 ug/mL Nic in 0.2% sacc)
Session 5 (200 ug/mL Nic in H_2O)

Supplemental Figure 4-1: No difference in total fluid intake during the nicotine two-bottle choice (2BC) paradigm between adolescent offspring from control and morphine-treated dams.

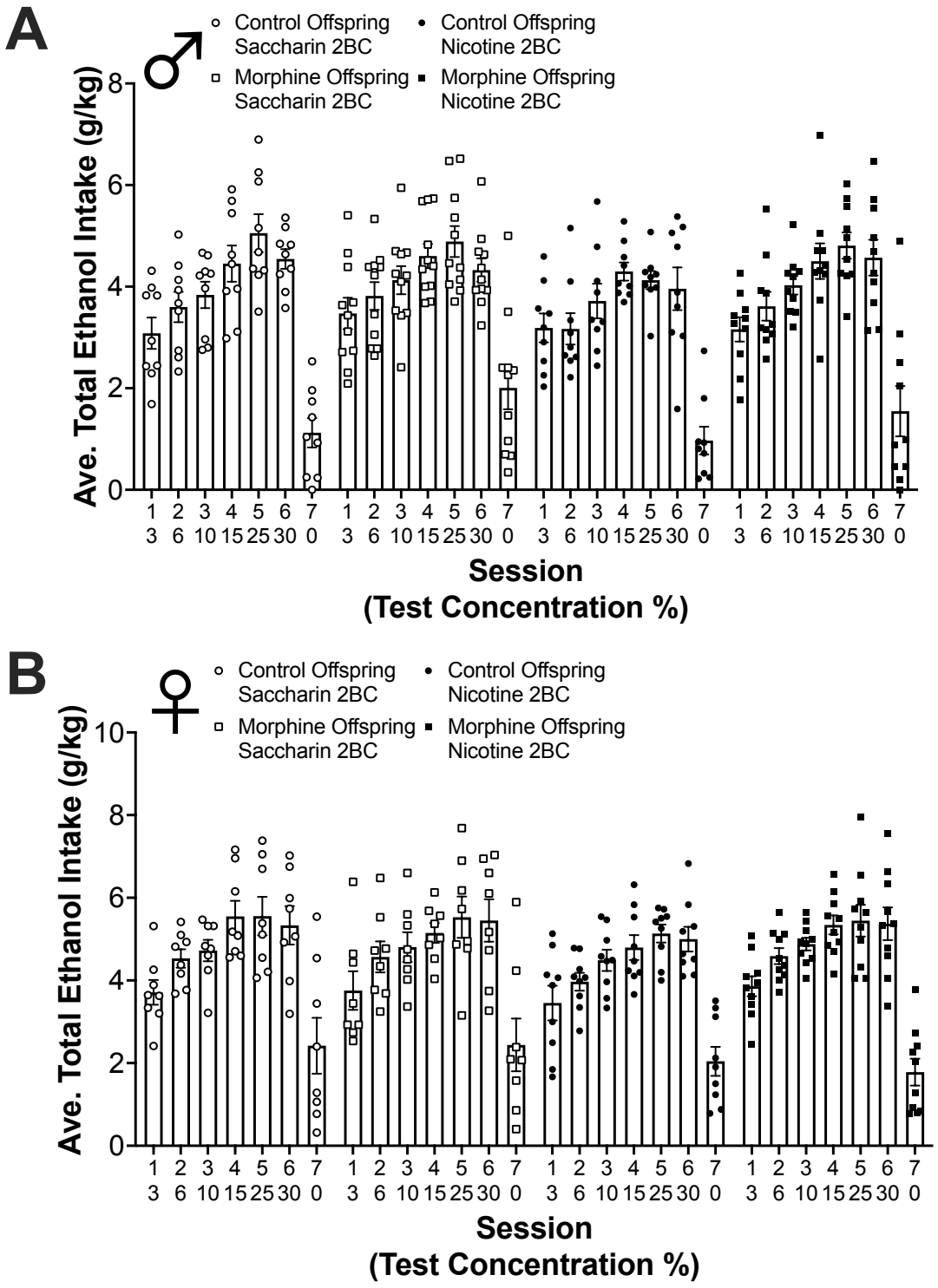
Total fluid consumption during the 2-hr (**A**) and 24-hr (**B**) nicotine 2BC paradigm during adolescence for offspring from control and morphine-treated dams (n=21,22). (**A**) Main effect of session (RM Two-Way ANOVA; $F(3.165, 129.8) = 32.80$, $p < 0.0001$). (**B**) Main effect of session (RM Two-Way ANOVA; $F(2.493, 102.2) = 121.0$, $p < 0.0001$).



Supplemental Figure 4-2: Investigation of adolescent offspring's taste perception of sucrose, saccharin, and quinine in a two-bottle choice (2BC) paradigm in offspring.

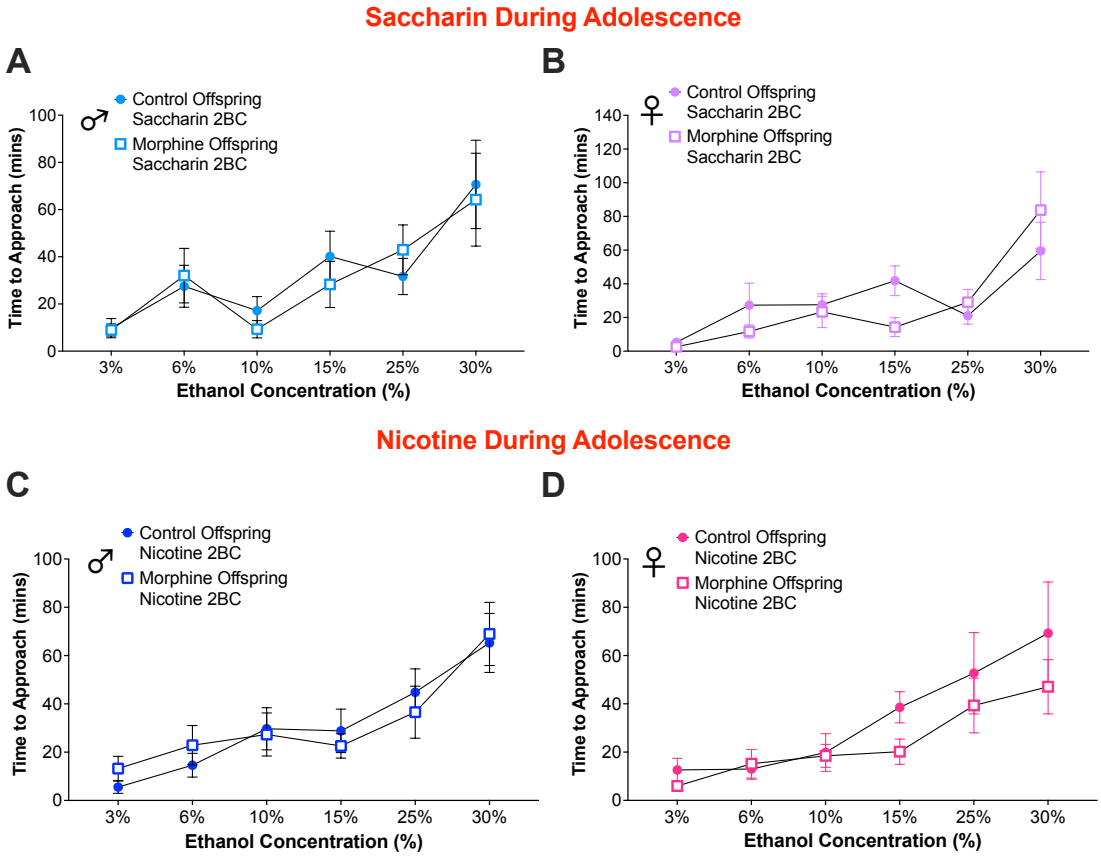
(**A-C**) Tastant preference for 1% sucrose (**A**), 0.2% saccharin (**B**), and 0.03 mM quinine (**C**) in a 2BC was determined for male (n=5-8) and female offspring (n=5-9) from control and morphine-treated dams. (**D-F**) Tastant intake for 1% sucrose (**D**), 0.2% saccharin (**E**), and 0.03 mM quinine (**F**) in a 2BC was determined for male (n=5-8) and female offspring (n=5-9) from control and morphine-treated dams.

* p<0.05, ** p<0.01, main effect of dam treatment or sex



Supplemental Figure 4-3: No difference in average total ethanol intake in adult offspring exposed to nicotine or saccharin 2BC during adolescence.

We investigated ethanol binge-like drinking in a limited access (4-hr) two-bottle choice (2BC) paradigm in offspring exposed to nicotine or saccharin 2BC during adolescence. The sum of the ethanol consumed from the test concentration bottle (3-30%) and the reference concentration bottle (20%) was calculated for each session. (**A & B**) Average total ethanol intake across sessions in male (n=9-11) (**A**) and female (n=8-10) (**B**) offspring from control or morphine-treated dams that were exposed to nicotine 2BC or saccharin during adolescence. (**A**) Main effect of concentration (RM three-way ANOVA; $F(4.532, 158.6) = 120.2$, $p < 0.0001$). (**B**) Main effect of concentration (RM three-way ANOVA; $F(4.208, 130.5) = 67.45$, $p < 0.0001$).



Supplemental Figure 4-4: No difference in the latency to interact with the ethanol bottle in adult offspring exposed to saccharin or nicotine 2BC during adolescence, measured using lickometers.

We investigated ethanol binge-like drinking microstructure using lickometers in a limited access (4-hr) two-bottle choice (2BC) paradigm in adult offspring exposed to nicotine or saccharin 2BC during adolescence. (**A & B**) Average time to approach the test

concentration (3-30% ethanol) bottle across sessions in male (n=9,11) (**A**) and female (n=8,8) (**B**) offspring from control or morphine-treated dams that were exposed to saccharin 2BC during adolescence. (**C & D**) Average time to approach the test concentration (3-30% ethanol) bottle across sessions in male (n=9,0) (**C**) and female (n=9,10) (**D**) offspring from control or morphine-treated dams that were exposed to nicotine 2BC during adolescence.

Main effect of concentration for male (RM three-way ANOVA; $F(2.942, 103.0) = 16.21$, $p < 0.0001$) and female offspring (RM three-way ANOVA; $F(2.647, 82.05) = 18.04$, $p < 0.0001$).

CHAPTER 5: General Discussion and Future Directions

Vanessa C. Fleites

5.1. Overview

Misuse of prescription and/or illicit opioids has led to an increase in opioid overdose deaths. There has also been a surge in the rate of pregnant women that use opioids for medical reasons and/or recreationally and develop Opioid Use Disorder. Consequently, this has led to a rise in the number of newborns born with Neonatal Opioid Withdrawal Syndrome (NOWS). Newborns with NOWS usually require longer hospital stays to reduce severity of symptoms with non-pharmacological and/or pharmacological interventions, which includes morphine for severe cases. As highlighted throughout this dissertation, opioid exposure can have detrimental effects on the mother-child dyad, as opioids can cross the placenta and affect the development of the central nervous system. Clinical studies have demonstrated that babies born to mothers with OUD have decreased body weight, score lower in cognitive assessments in early childhood, and display signs of aggression and impulsivity (Weller et al., 2020). In addition, meta-analyses have revealed that children between ages 3 months – 15 years old that were exposed prenatally to opioid maintenance therapies (OMT) display impairments in psychomotor, behavioral, attentional, and executive function (Andersen et al., 2020). Longitudinal studies are rare, and those that exist have reported that adults exposed to *in utero* opioids are more likely to have a depressive episode, Attention Deficit Hyperactivity Disorder (ADHD), have more sexual partners, and abuse alcohol in their lifetime (Nygaard et al., 2020). However, there are still large gaps of knowledge related

to the long-term behavioral consequences of *in utero* opioid exposure, including lifetime risk of drug misuse, and whether there are vulnerable and/or resilient sub-populations among those exposed to opioids during development. Considering that most preclinical models use injections, osmotic minipumps, or forced oral access to opioids as exposure paradigms for the dyads, the first step in my thesis was to validate a volitional oral morphine self-administration model that has face validity and limits the amount of stress experienced by the dams (Chapter 2). Using this paradigm as the basis for the studies described in Chapters 2-4, I identified impairments in neonate, adolescent, and adult offspring behavior, that are sex- and age- dependent and also depend on the length of opioid exposure paradigm used for the dyad. These results add to the growing body of preclinical literature on the effects of maternal opioid exposure on offspring behavior. To our knowledge we were the first to investigate the effects of maternal morphine exposure on nicotine and alcohol use. In this chapter, I review our findings with special consideration on the advantages and limitations of the studies and future directions of our work.

5.2. Developing a translational maternal opioid exposure model

Morphine is commonly given to reduce pain in pregnant women. For example, urine screening in pregnant women using opioids revealed that ~26% of them used morphine, among other opioids, such as methadone (~31%) and buprenorphine (~5.3%), both of which are commonly given as OMTs (Skumlien et al., 2020). In addition, newborns experiencing severe symptoms of NOWS are also given morphine to reduce symptoms. Therefore, it is important to investigate the effects of *in utero* morphine exposure using a

translationally relevant model. Preclinical models that have studied the effects of *in utero* opioid exposure on offspring outcomes have used daily injections, osmotic minipumps, and forced oral drug exposure (see Appendix Tables 1-3). With these methods, however, it is hard to distinguish the effects solely due to maternal drug exposure from those due to an interaction of stress (injections and/or withdrawal) and the opioid administered. For this reason, we validated the continuous oral morphine paradigm described in Chapter 2. Dams were first made dependent on one bottle of morphine in a sweet solution, and then were transitioned to a 2BC where they received one bottle of morphine in a saccharin solution, versus one bottle of water. Many studies using 2BC models increase the morphine concentration across sessions and add quinine - a bitter tastant – to the control solution to mimic the bitter-like taste of morphine in the second bottle (Belknap et al., 1993; Eastwood & Phillips, 2014; Ferraro et al., 2005). Instead of adding another tastant (quinine) that could change dams' taste perception, we decided not to add saccharin to the control water bottle. In this way, only the morphine bottle would be sweetened and the saccharin would increase the palability of the morphine bottle. In summary, we have established a paradigm that delivers opioids per os (p.o), the route commonly used for opioid self-administration, that is associated with low levels of stress because morphine is available *ad libitum*. Such paradigm successfully induces drug dependence in the dams (as assessed in Figure 2-2).

An important consideration is that 80-95% of pregnant women with OUD report tobacco use and about 35% cite simultaneous cannabis, cocaine and benzodiazepines use (Tobon et al., 2019). To increase the translational value of our maternal opioid exposure paradigm, future work can include exposure to additional drugs (for example a 3BC paradigm with opioid, nicotine, and saccharin) to provide a maternal poly-drug

treatment. This type of work will allow to determine whether the effects seen in clinical studies are due to an additive or synergistic effect between opioids and other drugs. It will take years for clinical studies such as those conducted under the umbrella of the Adolescent Brain Cognitive Development℠ (ABCD) Study to provide information on the long-term effects of *in utero* exposure to opioids; thus, preclinical studies such as those described in this thesis can provide important mechanistic insight and inform the human experimentation.

5.3. Opioid pharmacokinetics in mothers and offspring

Morphine is the active metabolite of heroin and is largely used in both adults and neonates for pain management and sedation pre- and post-operatively. Pain control continues to be the accepted method for morphine dosing, especially in pre-term babies and in newborns experiencing NOWS (Pacifici, 2016). Morphine undergoes first-pass metabolism where almost 90% of morphine is eliminated, which means doses given orally must be six-fold larger than those given intravenously to achieve similar analgesic effects (Pacifici, 2016). The elimination half-life of oral morphine (30-40 mg) is 2-3.5 hours in adults, while it is much more variable and rapid in newborns (~6.5 +/ 2.8 hours) (Lugo & Kern, 2002; Pacifici, 2016). To experience pain relief in neonates, blood level concentrations must reach ~120 ng/mL and must not go over 300 ng/mL as this is when overdose symptoms begin to appear (Pacifici, 2016).

Similarly in a preclinical setting, the morphine dose given to rodents is variable. When mice were tested in nociceptive tests, the median effective dose (ED_{50}) for morphine was ~ 10 mg/kg i.p. (Raffa et al., 1992), 3-20 mg/kg s.c. (Sora et al., 2001),

and 15-20 mg/kg p.o. (Nickel, 1987). In rodents, a 20 mg/kg/day morphine dose is 3.2 times the human daily dose of 60 mg based on body surface area (Pfizer, n.d.). To increase the face validity of our studies, our criteria in creating a volitional oral morphine treatment for dams was for dams to drink daily morphine doses of at least 20 mg/kg (Figure 2-2, Figure 3-1), since this dose has been shown to produce analgesia in rodents and would align with the human literature on using a dosage that mitigates pain.

In clinical settings, the pharmacokinetics of morphine varies depending on the medical complication the neonate is experiencing, and if the neonate is preterm or full-term (Pacifici, 2016). This is particularly important considering that opioids, including morphine, are given to neonates if they are experiencing severe symptoms of NOWS. In fact, a higher percentage of morphine stays free (not bound to plasma protein) in neonates than in adults, which means a greater percentage of morphine can enter the neonate's brain, possibly leading to the increased sensitivity to morphine in neonates (Pacifici, 2016). The dose of opioids given to both pregnant mothers and neonates, and how much is found in circulation, are important considerations in how they can affect offspring in the future, including developmental and behavioral outcomes, and how opioid levels in the periphery/brain can affect vulnerability/resiliency in this population. This is an important consideration for preclinical research as well; however, very few studies have reported how much opioid is found in the plasma and brain of the dyad. Future work in the De Biasi lab aims to address this limitation of our own studies, as we are interested in investigating whether the offspring's behavioral outcomes correlate with their plasma and brain morphine levels after lactation. Interestingly, when we correlated dams' morphine dose (mg/kg) with offspring behavioral outcomes (Table 2-1), we found the direction of correlation to be opposite to what we expected, suggesting that plasma

and brain opioid levels might serve as a better metric to understand offspring behavior variability.

One preclinical study reported the circulating levels of morphine in both dam and offspring after *in utero* opioid exposure and showed that opioids can be detected in both plasma and brain of the offspring. In that study, drug-naïve pregnant dams were injected during gestation day (GD) 17 with heroin (2.5 μmol/kg, s.c.), and morphine was detected not only in maternal blood and brain, but also in fetal blood and brain at levels similar to those in the dam (Kvello et al., 2019). This suggests that our future studies should also find detectable levels of morphine in both dams and offspring, and that levels in the plasma and brain might inform us on why certain offspring are classified as having a more severe behavioral phenotype than others, as shown in Chapters 2-3. Furthermore, our work will benefit from the data emerging from studies that use pharmacokinetic modeling and simulation as a tool to inform optimal opioid dosing regimens in adults (Liu et al., 2019) and in newborns experiencing NOWS (J. N. Moore et al., 2018; Tang et al., 2021; van Hoogdalem et al., 2022), as those data will help us estimate how much of the drug reaches the offspring's periphery and brain.

5.4. Effect of withdrawal in both dams and offspring drives adverse outcomes in offspring

Opioid exposure can cause withdrawal upon cessation of drug taking in both clinical and preclinical models (Papaleo & Contarino, 2006). Studies have reported that methadone withdrawal is more severe than heroin withdrawal, and that children exposed to *in utero* methadone have more behavioral problems, including inability to control their

anger, compared to children that were exposed to heroin *in utero* (Davis & Templer, 1988). This begs the question of whether vulnerability to long-term adverse outcomes not only depends on which type of opioid offspring are exposed to, but also the potential differences in withdrawal severity. For example, in Chapter 2 we cross-fostered the offspring to a drug-naïve dam on PND 7, where they potentially would experience drug withdrawal from not lactating from their drug-treated dam. We find that the withdrawal after PND 7 leads to characteristics similar to those observed in newborns with NOWS, including high-pitched crying, increased adolescent/adult anxiety-like behavior, and changes to ethanol intake in a sex-specific manner (Chapter 2). This highlights how morphine withdrawal in our prenatal-perinatal maternal paradigm might confer important neurochemical and behavioral changes that lead to worst outcomes in offspring, including having a higher percentage of offspring classified in the 'high' behavioral severity phenotype, as shown in Figure 2-5.

Preclinical studies have also reported variability in the behavioral responses to protracted withdrawal from morphine in mice (Bravo et al., 2020; Buckman et al., 2009). The withdrawal profile for morphine in rodents can be very different among neonates, adolescents, and adults (van den Hoogen et al., 2021). In addition, a preclinical study showed that precipitated withdrawal from morphine leads to sensitization of the mesolimbic dopamine system and significant changes in gene expression in the nucleus accumbens and dorsal striatum, which was not as apparent in mice that stayed on a continuous morphine paradigm (Lefevre et al., 2020). The changes that occur due to drug withdrawal, like in our dams, can affect neurocircuitry and downstream signaling pathways differently than a continuous opioid exposure, and this potentially can affect the offspring. Interestingly, in Chapter 2 (Table 2-1) we report that dams' total number of

physical signs during spontaneous withdrawal from morphine after the first week of the C2BC paradigm positively correlated with adolescent offspring's total number of marbles buried, so the higher the number of somatic signs displayed by dams during withdrawal, the more marbles offspring buried, or the more anxiety/compulsive-like behavior the offspring displayed. This is especially interesting since out of all the behavioral tests administered during adolescence in offspring exposed to prenatal-perinatal morphine, this was the only one where we saw a significant effect of dam treatment, where adolescent offspring displayed increased anxiety/compulsive-like behavior compared to control offspring (Figure 2-4).

Currently there is debate on whether mothers should be tapered off opioids during pregnancy, and whether newborns should not be given opioids to reduce NOWS severity. Based on the vast evidence that withdrawal can be detrimental to the dyad, studies have begun to identify alternative non-opioid therapeutics that might be used and that do not confer additional side-effects. Future work in our prenatal-perinatal morphine exposure studies can have non-opioid therapeutics administered to dams and/or offspring after withdrawal (e.g. after cross-fostering on PND 7) to determine whether this approach ameliorates the behavioral deficits we reported in Chapter 2.

Potential non-opioid therapeutics that could be administered to the dyad to reduce withdrawal symptoms are drugs that reduce pro-inflammatory signaling. A study by McClung and colleagues reported that chronic morphine exposure and precipitated withdrawal led to specific changes in gene expression, and that morphine withdrawal actually led to a larger number of changes, which included genes involved in stress and immune responses (McClung et al., 2005). Increased microglia activation and induction of pro-inflammatory cytokine secretion have been implicated in morphine dependence,

withdrawal, and tolerance (Eidson & Murphy, 2019; Thomas et al., 2022; Y. Zhang et al., 2011). One study revealed that prenatal-perinatal methadone exposure has long-lasting effects in the neonatal inflammatory response, both in the periphery and in the brain (Jantzie et al., 2020). This hyperactive inflammatory response during early PNDs in rats exposed to methadone during gestation and throughout lactation was also associated with impaired cognition in these offspring (Jantzie et al., 2020). Targeting the inflammatory response in newborns experiencing severe NOWS might be able to reduce symptom severity and mitigate potential behavioral deficits later in life. Studies have already shown that decreasing neuroinflammation and proinflammatory signaling using phosphodiesterase inhibitors can improve cognition and decrease pain, among other benefits in various disease states (Pearse & Hughes, 2016; Sugin et al., 2020; F. fang Zhang et al., 2022). Specifically, phosphodiesterase 4B (PDE4B) inhibitors, like A-33, which decreases inflammation, could be a novel therapeutic for treating newborns exposed to prenatal opioids that are undergoing NOWS, as it has been shown to ameliorate cognitive deficits after traumatic brain injury (Wilson et al., 2017), decrease ethanol binge-like drinking (Leonardo Jimenez Chavez et al., 2021), decrease methamphetamine self-administration (Honeywell et al., 2022), and decrease depressive-like behavior (C. Zhang et al., 2017). PDE4B has also been shown to be associated with anxiety-related disorders in humans, and anxiety-like behavior in mice in the PFC and hippocampus (Meier et al., 2019). Reducing immune activation is a possible avenue to pursue, because a drug like A-33 could be given to newborns as an alternative to opioids to dampen NOWS symptoms. This approach might decrease withdrawal symptoms at an early stage and might be “protective” over time. This hypothesis could be investigated in our prenatal-perinatal morphine exposure model to determine whether reducing proinflammatory signaling using A-33 in cross-fostered

offspring could reverse the behavioral impairments reported, and whether this treatment would cause a higher percent of offspring from morphine-treated dams to classify under the 'low' global behavioral score classification instead of the 'high' category.

5.5. Interaction of ethanol and opioids and potential sex-specific effects on offspring

It is well documented that there are ethanol-opioid interactions and opioid receptors are implicated in ethanol reward (Drews & Zimmer, 2010). This is elucidated by the fact that an opioid receptor antagonist, naltrexone, is used to treat Alcohol Use Disorder (Oslin et al., 2006). In addition, acute ethanol stimulates the release of β-endorphins in reward-related brain regions (Jarjour et al., 2009). Chronic ethanol exposure, however, decreases β-endorphin release and opioid receptor activity in various brain regions (Drews & Zimmer, 2010; Hermann et al., 2017; Przewłoka et al., 1994; Sarkar et al., 2007). These effects depend on the ethanol exposure paradigm and ethanol dose used, among others. In Chapters 2 and 3, I used the ethanol intermittent two-bottle choice (2BC) paradigm to assess volitional drinking in offspring exposed to either prenatal-perinatal morphine exposure (Chapter 2) or prenatal-preweaning morphine exposure (Chapter 3). Considering that previous studies have shown that offspring exposed to *in utero* morphine have altered opioid receptor levels and activity (Chiou et al., 2003; Schindler et al., 2004; Šlamberová et al., 2003; Vathy et al., 2003), the decrease in ethanol intake in male offspring (Chapter 2) and the decrease in ethanol preference in female offspring (Chapter 3) could be due to baseline reductions in the system's activity. For example, Chiou *et al.* (2003) showed that offspring had decreased MOR density in

multiple brain regions, including the striatum, after exposure to a maternal morphine paradigm that started pre-conception and extended throughout lactation, which was similar in length to the maternal morphine exposure model we used in Chapters 3 and 4. In addition, ethanol treatment and/or withdrawal decreases mRNA levels and density of MORS in a brain-region specific manner (Hermann et al., 2017; Turchan et al., 1999; Winkler et al., 1998). Therefore, if the offspring used in our studies already had lower levels of MORs due to *in utero* morphine exposure, ethanol treatment might lead to phenotype we observed (decreased alcohol preference or intake).

Indeed, rats bred to selectively display higher ethanol intake and preference ("ethanol-preferring") have a greater density of MORs in the nucleus accumbens (NAc), among other brain regions (Hyytiä et al., 1999; McBride et al., 1998). In addition, infusions of MOR antagonists, which preferentially bind to MORs, into the NAc and ventral tegmental area (VTA), reduced lever pressing by blocking MORs (June et al., 2004). Reduced alcohol drinking is also observed when MOR antagonists are injected, and in MOR knockout mice (Contet et al., 2014; Hall et al., 2001; Lasek et al., 2007; Morales et al., 2020). Together, these data support the notion that decreased ethanol intake in male offspring exposed to prenatal-perinatal morphine (Chapter 2) and decreased ethanol preference in female offspring exposed to prenatal-preweaning morphine (Chapter 3) might be due to decreased opioid receptor levels and activity following maternal morphine.

In a clinical study, authors found a significant association of proopiomelanocortin (POMC) haplotypes specifically in a female German population, suggesting that β-endorphin might be important for gender-related differences in alcohol use disorder (Racz et al., 2008). Also, a greater effect was observed in female MOR knockout mice

which drank less ethanol in a 2BC paradigm and also showed less ethanol-induced CPP (Hall et al., 2001; Racz et al., 2008). Although decreased ethanol preference and intake might seem protective for the offspring, we should be cautious in drawing that conclusion because the effect of stress and drug use during critical periods, including adolescence, might trigger maladaptive behavior in offspring by "pushing" the system. This is demonstrated in Chapter 4, where the interaction between prenatal-preweaning morphine exposure and nicotine intake during adolescence drives female offspring to escalate ethanol binge-like drinking at various concentrations during adulthood. Furthermore, clinical and preclinical data also suggest that stress through early life adversity or traumatic stressful events during critical developmental periods can enhance the risk for developing adverse outcomes, including psychiatric diseases and future drug use (Avishai-Eliner et al., 2002; Gur et al., 2019; Levis et al., 2021; Luby et al., 2020).

It is also important to contemplate the importance of individual behavioral differences, considering that using an antagonist/agonist of delta opioid receptors (DORs) has differential effects depending on whether rodents have a low-drinking or high-drinking ethanol phenotype (Margolis et al., 2008; Mitchell et al., 2012). For our studies, this could speak to the potential existence of vulnerable, sex-specific, sub-populations that depend on the maternal paradigm used. For example, in the prenatal-perinatal morphine exposure model (Chapter 2), a larger proportion of male offspring had a high, or "worst", severity behavioral phenotype, while in the prenatal-preweaning morphine exposure model (Chapter 3), female offspring seemed to be the vulnerable sub-population. Identifying vulnerable sub-populations by looking at behavior holistically, as is done in a clinical setting (T. M. Moore et al., 2020), will be important for identifying

pharmacological targets for better therapeutic intervention, as discussed in a later section.

There is some clinical evidence that males are more likely to develop NOWS (Charles et al., 2017), and/or develop more severe symptoms (Jansson et al., 2007). Boys exposed to *in utero* opioids displayed poorer cognitive and language development, compared to non-exposed boys, although some studies show that the scores still remain within normal range (Nygaard et al., 2015; Skumlien et al., 2020). However, the results are mixed, as another study found cognitive deficits in females that persisted longer than those in males (Nygaard et al., 2015). This is in line with our own contradictory results showing that the vulnerable sub-population in Chapter 2 are male offspring, while they are female offspring in Chapters 3-4. For example, we saw a decrease in ethanol intake in male offspring (Chapter 2) and a decrease in ethanol preference in female offspring (Chapter 3). Those differences are likely due to the different lengths of morphine exposure used for each study and/or the severity of withdrawal symptoms experienced by the offspring.

A possible explanation for why male offspring from morphine-exposed dams are a more vulnerable sub-population, compared to female offspring, in the cross-fostered paradigm used in Chapter 2, could be due to morphine's widely known ability to decrease testosterone levels and other reproductive hormones through the Hypothalamic-Pituitary-Gonadal (HHG) axis in both human and rodent males (Ajdary et al., 2021; Eichenbaum et al., 2015; Marudhai et al., 2020; Wehbeh & Dobs, 2020; Yilmaz et al., 1999). Testosterone levels peak in male rodents between birth-PND 7 (Clarkson & Herbison, 2016; Turano et al., 2019), but not in females. If morphine decreases testosterone levels at a critical period in males, i.e. when they should have

high testosterone levels (~PND 7), the phenomenon could have long-lasting effects, and could explain the sexually dimorphic changes observed in our paradigm where offspring are cross-fostered at PND 7. Also, in a study where rats were injected with a low dose of morphine (2mg/kg) from GD 13-20, MOR binding increased on PND 1 and PND 7 (Bhat et al., 2006), which coincides with the timeframe when testosterone levels peak and when our offspring are cross-fostered. This could lead to the male-specific deficits in behavior we report in Chapter 2. In addition, a study using a slightly similar paradigm to the one we used in Chapters 3-4, with dams exposed to morphine from pre-gestation until offspring PND 10, reported alterations in reproductive behavior and lower levels of ovarian estradiol and progesterone in female offspring (Siddiqui et al., 1997). Hence, differences in the length of maternal opioid exposure paradigms can yield paradigm-specific changes in the offspring.

5.6. Nicotine exposure during adolescence has long-lasting effects that lead to changes in adult ethanol binge-like drinking

Tobacco/nicotine use during adolescence is a strong predictor for problems with alcohol later in life (Riala et al., 2004). Nicotine acts on nicotinic acetylcholine receptors (nAChR), while ethanol acts on various neurotransmitter receptors including N-methyl-$_D$-aspartic acid (NMDA), gamma-aminobutyric acid (GABA), and partially, nAChRs. Although these two drugs largely act on separate neurotransmitter systems, they can indirectly affect common neurochemical systems, including the endogenous opioid system. Acute nicotine exposure leads to the release of enkephalins, while chronic nicotine treatment leads to a reduction in enkephalin levels in rodents (Houdi et al.,

1991, 1998). The data on whether nicotine upregulates and/or downregulates MORs are mixed, and may be dependent on the brain region, nicotine dose, and nicotine state (sated vs. withdrawal) (Drews & Zimmer, 2010).

In addition, there is cross-tolerance between nicotine and morphine (Drews & Zimmer, 2010). One study showed that opioid receptor activity is important in the expression of precipitated-withdrawal signs in nicotine-dependent animals, as somatic signs can be reduced by morphine and precipitated by naloxone (Malin et al., 1993). Another study showed that nAChRs are important for the expression of morphine somatic withdrawal signs (Muldoon et al., 2014). In addition, knockout studies have shown that MORs are important for nicotine-induced CPP, precipitated withdrawal signs, and analgesic tolerance (Berrendero et al., 2002; Galeote et al., 2006; Walters et al., 2005). Chronic morphine exposure can alter acetylcholinesterase activity and levels of nAChRs in brain regions important for the manifestation of withdrawal signs (Neugebauer et al., 2013). Together, these observations highlight the importance of opioid receptor activity for nicotine withdrawal signs, possibly more so than for nicotine volitional self-administration, considering that the role of opioids receptors in nicotine intake and preference is unclear. In addition, in line with our hypothesis that the offspring in our studies have decreased MOR opioid receptor and peptide levels, and that opioid receptor activity might be more important for withdrawal than nicotine oral self-administration, these observations could also explain why no apparent differences in nicotine intake and preference during adolescence were seen in our offspring (Chapter 4). Indeed, chronic nicotine can alter KOR function in adult -but not adolescent- rodents, which is important considering that KOR activation and blockade manipulated withdrawal signs after nicotine exposure (Tejeda et al., 2012). Because opioid receptors seem to be

especially important for signs of withdrawal from nicotine, future work in our studies should evaluate whether offspring exposed to *in utero* opioids have altered physical signs of nicotine withdrawal, which could have implications for drug relapse and craving.

Kappa opioid receptor (KOR) signaling has been shown to be important for dysphoric states in addiction-related behaviors (Laurence Lalanne et al., 2014). Prodynorphin gene deficient mice display no changes in nicotine-induced CPP, but self-administered lower doses of nicotine, showing that the absence of dynorphin is important for the sensitivity to nicotine (Galeote et al., 2009). In addition, KOR knockout mice have lower ethanol intake (Kovacs et al., 2005). Specifically female mice lacking the preprodynorphin gene display lower ethanol preference in a 2BC (Blednov et al., 2006). These knockout mice also display changes in taste perception (Blednov et al., 2006; Kovacs et al., 2005), which could potentially explain our preliminary taste perception studies in Chapter 4 (Supplemental Figure 4-2). Furthermore, changes in KOR/dynorphin system in the offspring from our studies could explain the subtle changes in the nicotine 2BC data described in Chapter 4, and the larger change in ethanol binge-like drinking, considering that KORs/dynorphin differentially affect taste perception, and nicotine and ethanol misuse.

A separate study examined protein and mRNA levels of opioid precursors in a specific region of the hippocampus in offspring exposed to morphine during mid-gestation (GD 11-18) and found decreased proenkephalin/met-enkephalin levels, but increased prodynorphin/dynorphin levels (Schindler et al., 2004). It still needs to be determined whether this change in mRNA levels is functionally relevant. In addition, an exposure model like ours that starts pre-conception and extends until lactation might lead to different changes in endorphin, enkephalin, and dynorphin peptide levels than

what was found by Schindler *et al.* (2004). Future experiments in our lab will assess opioid receptor and endogenous opioid peptide levels after both maternal morphine paradigms (prenatal-perinatal and prenatal-preweaning), in baseline conditions and after volitional nicotine and ethanol administration to support our mechanistic hypothesis.

Sex differences have been found in cigarette smokers (Gan et al., 2008; Zeman et al., 2002; S. Zhang et al., 2017). For example, abstinent women have a higher likelihood of relapsing due to increased negative mood symptoms and craving (Xu et al., 2008). In addition, when estradiol levels are high during nicotine abstinence, females show increased craving and relapse rates (Wetherill et al., 2016). Intact female rats display increased anxiety-like behavior and associated gene expression changes, however this was not observed in ovariectomized female rats (Torres, Pipkin, et al., 2014). The phenomenon is interesting, considering that our results in Chapter 4 reveal female-specific changes in how maternal opioid exposure interacts with adolescent nicotine use to influence subsequent adult ethanol drinking. Nicotine withdrawal produces less affective and somatic changes during adolescence than during adulthood in female rodents (Torres et al., 2013), so this and the role of ovarian hormones during nicotine exposure/withdrawal could also help explain the subtle effects on nicotine intake during adolescence in our offspring in Chapter 4. Moreover, if underlying neurocircuitry was changed by adolescent nicotine exposure, the effect could be more pronounced in adulthood, when we see increased ethanol intake at specific concentrations in female offspring exposed to nicotine during adolescence after prenatal-preweaning morphine exposure (Figure 4-3). Furthermore, one study reported that ovarian hormones are important for ethanol's reinforcing effects in a dose-dependent manner, where female

rats showed ethanol-CPP, although this was not observed in ovariectomized female rats or males (Torres, Walker, et al., 2014).

Together, these results support our hypothesis that prenatal-preweaning morphine exposure might alter KOR/dynorphin system activity in our offspring. Those potential receptor changes together with subtle changes in ovarian hormones induced by morphine *in utero* and nicotine during adolescence, could underlie increased ethanol intake in female offspring during adulthood.

5.7. Hypothalamic-Pituitary-Adrenal (HPA) axis functionality changes and potential effects on behavior

The role of *in utero* morphine exposure on HPA axis functionality and subsequent response to various stressors in a clinical setting is largely unknown. However, preclinical studies have elucidated that offspring exposed prenatally to morphine have a hypoactive stress response, exemplified by decreased stress-induced hormone levels and altered behavioral responses (Klausz et al., 2011; Lesage et al., 2000; Rimanóczy et al., 2003; Šlamberová et al., 2004). It is of particular concern how the hypoactivity of the HPA axis in offspring exposed to *in utero* opioids is behaviorally manifested when other confounds are added, like drug misuse during adolescence, stressful environmental living conditions, and childhood trauma, etc. There could be long-lasting implications if such hypoactive HPA axis phenotype were found in clinical settings, especially considering that some of the children born to mothers using opioids experience highly stressful environments, either living at home with parents who misuse drugs, or in foster care homes (Lejeune et al., 2006; Nygaard et al., 2020; Sarfi et al.,

2021). Especially considering that early-life adversity alters stress-sensitive neurons and peptide levels, including corticotropin-releasing factor (CRF), in brain regions important for stress- and reward-related behaviors, and is a potential mechanism for risk and resiliency phenotypes (Avishai-Eliner et al., 2002; Birnie et al., 2020; Singh-Taylor et al., 2015).

The type, duration, and intensity of the stressor need to be considered when studies investigating the effects of stress and ethanol misuse contradict each other. The data presented in Chapter 2 highlights how male offspring that had prenatal-perinatal morphine exposure did not habituate to the second acute restraint stressor and continued to have lower ethanol intake compared to their baseline intake. In contrast, after the second stressor, male control offspring drank ethanol doses comparable to their pre-stress drinking baseline. In addition, we did not see changes in corticosterone levels among offspring after we administered the dexamethasone suppression test to study the functionality of the feedback mechanism of the HPA axis (Supplemental Figure 2-3), suggesting that the inability to habituate to the second stressor in offspring from morphine-exposed dams was not due to impairments in the feedback loop of the HPA axis. Future work should investigate changes in ACTH and CRF, as immediate downstream regulators of the HPA axis in offspring from morphine-treated dams. As mentioned in the Introduction (Section 1.5.3) and Discussion thus far, many preclinical studies found decreased ACTH levels in offspring exposed to *in utero* morphine after various types of stressors, including restraint stress (Klausz et al., 2011; Lesage et al., 2000; Rimanóczy et al., 2003; Šlamberová et al., 2004). However, not all these studies found decreased levels of corticosterone after a stressor. This is similar to preliminary data where we report unchanged corticosterone levels from offspring exposed to

prenatal-preweaning morphine exposure. As shown in Figure 5-1, we did not detect changes in corticosterone levels between offspring from morphine-treated dams and control dams, either at baseline or while ethanol sated. However, our data show that ethanol-dependent control offspring have lower corticosterone levels compared to control offspring that only received water (basal state), while in ethanol-dependent offspring from morphine-treated dams we did not observe such reduction in corticosterone (Figure 5-1). This result further highlights the complexity of the opioid-ethanol interaction occurring in our offspring, where we hypothesize that possible changes in opioid receptor/peptide activity due to *in utero* morphine exposure alters the offspring's HPA axis functionality, and this in turn, affects ethanol use risk.

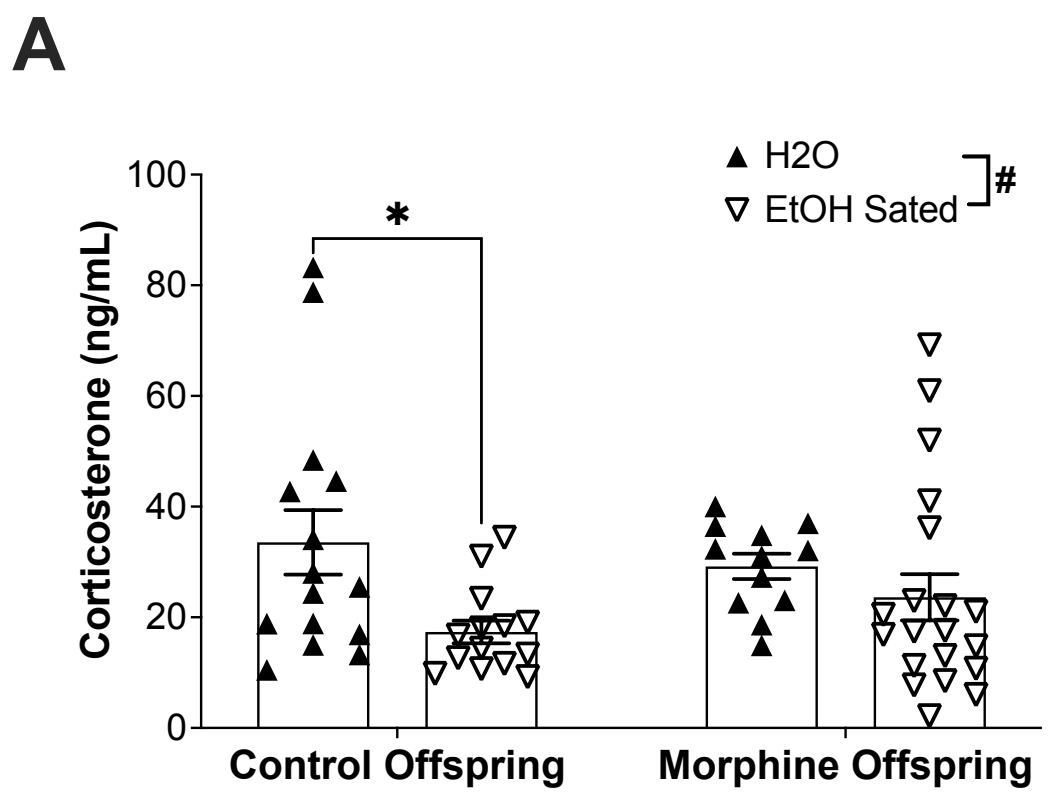


Figure 5-1: Preliminary data reveals that prenatal-preweaning morphine exposure alters ethanol's ability to decrease corticosterone levels in offspring.

(A) Adult offspring exposed to prenatal-preweaning morphine, as described in Chapter 3, were single-housed in adulthood and were either maintained on water (baseline) or were administered the 6-week ethanol intermittent two-bottle choice paradigm. At the end of the 6-weeks, trunk blood was collected from all offspring and corticosterone levels were analyzed during baseline (H_2O) and while ethanol sated. A Two-Way ANOVA revealed a main effect of state (basal or ethanol sated) (Two-Way ANOVA; $F(1, 57) = 6.556$; $p = 0.0131$), but no effect of dam treatment or interaction of both variables. Sidak post-hoc analysis revealed that corticosterone levels were significantly reduced while control offspring were ethanol sated, compared to control offspring at basal states. However, this ethanol-induced suppression of corticosterone was not seen in offspring from morphine-treated dams.

# $p<0.05$, main effect of state (basal or ethanol sated); * $p<0.05$, post-hoc analysis

It has been proposed that opioid receptor antagonists can reduce drug consumption and craving due to their effects on the HPA axis (Ray et al., 2009; Roche et al., 2010; Schluger et al., 1998). In addition, the efficacy of naloxone in treating AUD in individuals with a polymorphism in the MOR gene, A118G, might be associated with HPA axis activation (Chong et al., 2006; Wand et al., 2002). This again highlights the complexity of the interaction of opioid and ethanol systems and how they might converge on modulating the stress response.

5.8. Using a global behavioral score as a predictor for vulnerable and/or resilient populations.

In Chapters 2 and 3, we integrated multiple behavioral outcomes into a composite score, referred to as global behavioral score (GBS). This allowed us to characterize offspring behavior holistically, an approach that has been used in multiple areas of research (El-Kordi et al., 2013; Guyenet et al., 2010; Möller et al., 2018; O'Neal et al., 2020; Pereira de Souza Goldim et al., 2020; Shahi et al., 2019). The use of a global

severity score classification system allowed us to examine the distribution of animals' performance across multiple behavioral tests, where higher values represent higher behavioral symptom severity. Assessing inter-individual differences to evaluate vulnerable and/or resilient populations based on behavioral outcomes and functional connectivity can provide valuable information. For example, the Adolescent Brain Cognitive Development (ABCD) study is a longitudinal study where neuroimaging and functional Magnetic Resonance Imaging (fMRI) tasks are completed during childhood (baseline) to assess changes during adolescent development (Chaarani et al., 2021). The children are followed for 10 years and the study investigates all the factors that can influence brain development, including substance use in the individuals and their parents' drug use history (Bjork et al., 2017; Hagler et al., 2019; Kwarteng et al., 2021). For example, the ABCD study has reported that high family conflict and limited parental monitoring is associated with behavioral problems and changes within specific brain regions, such as the anterior cingulate cortex, in children (Gong et al., 2021). In addition, another ABCD study showed that the number of negative life events positively correlates with stronger negative cortico-limbic resting state functional connectivity in children (Brieant et al., 2021). This reveals that changes in children's behavior and brain functionality are particularly influenced by stressful life events and home environment. This is also true for children exposed prenatally to opioids, who have worst outcomes if they are in a foster home compared to control children in foster homes (Sarfi et al., 2021). Studies like the ABCD will provide a wealth of knowledge for understanding the effects of *in utero* opioid exposure on offspring behavior.

Based on our results in Chapters 2-4, brain dimorphism might be important when trying to identify vulnerable and resilient subpopulations. Using resources like the ABCD

study, which has already proven to be able to identify brain regions that can accurately classify biological sex in adolescent males and females, will be critical for future work analyzing data from children exposed to gestational opioids (Brennan et al., 2021).

Our results in Chapter 3 highlight how identifying differentially expressed genes (DEG) in offspring exposed to prenatal-preweaning morphine might help identify novel players important for inter-individual differences. For example, we identified carbonic anhydrase 13 as being an upregulated DEG in the PFC of female offspring from morphine-treated dams (Figure 3-3). Considering that carbonic anhydrase 13 is important for myelination, and hyper-myelination can lead to difficulty in extinguishing memories (i.e. PTSD), further understanding its role in the developing brain exposed to opioids will be important. The ABCD study revealed that variations in myelination and white matter microstructure might underly the variability in executive functions, which is important because of the associated risk with psychopathology and substance use (Cardenas-Iniguez et al., 2022).

It should also be considered that not every newborn exposed to *in utero* opioids develops NOWS. For example, one study reported that only 32% of newborns exposed to opioids prenatally displayed characteristic signs of NOWS (Skumlien et al., 2020). As mentioned in the previous chapters of this dissertation, this posits the question of whether the newborns that experience NOWS would be more vulnerable to develop psychiatric disorders and substance use disorders later in life. Their vulnerability and/or resiliency might depend on the severity of NOWS symptoms they experienced or even the type of opioid used during pregnancy. For example, one study advised that outcomes of children exposed to *in utero* opioids be segregated based on the type of opioid the mother used, especially because there might be differential effects on

behavioral outcomes based on whether children were exposed to “heavy” opioids, like heroin or fentanyl, or “light” opioids, like codeine, during gestation (Davis & Templer, 1988). This approach might reveal individual variability that could be important for therapeutic efficacy. Assessments made to identify risk and resiliency (T. M. Moore et al., 2020) in this population could be used in conjunction to this approach.

Considering that the effectiveness of pharmacological interventions is very variable, some studies have investigated how inter-individual differences might be a better predictor for behavior and drug efficacy. Many psychiatric diseases, like AUD, are heterogenous disorders where individuals vary on symptomology and withdrawal severity. The US Food and Drug Administration (FDA) has also proposed to embrace this heterogeneity and stratify subjects based on group characteristics that would better allow for the detection of a medication’s effect (FDA, 2019). This idea has also been proposed in preclinical studies by clustering subjects based on withdrawal severity (Quijano Cardé & De Biasi, 2022), and/or by using a composite score to identify behavioral phenotypes (O’Neal et al., 2020). For example, one preclinical study revealed that although prenatal stress did not alter baseline ethanol preference, when inter-individual differences in pre-stress ethanol baseline were considered and animals were divided into ‘high-preferring’ and ‘low-preferring’, an effect of stress on ethanol preference was detected (Darnaudéry et al., 2007).

We hope this new mindset – identifying inter-individual differences - is used more widely in both preclinical and clinical research, as it could lead to more efficacious drugs and bring new knowledge on the mechanisms underlying these differences.

5.9. Concluding Remarks

Both clinical and preclinical studies investigating the effects of *in utero* opioid exposure have found contradictory results. Due to cost and time constraints, there is still much that is not known about the consequences of dyad opioid exposure. Collectively, the results in this dissertation shed light on behavioral alterations in neonate, adolescent, and adult offspring using two maternal morphine exposure models that confer sex-specific differences in baseline behavior and drug misuse liability. This dissertation highlights how analyzing behavior holistically will give novel insight in vulnerable/resilient sub-populations in offspring, a fact that could have translational value.

APPENDIX

Appendix Table 1. Most commonly cited pre-clinical studies in our review investigating the effects of full gestational-only morphine on offspring outcomes.

Reference	Animal model	Parental route of administration	Offspring age of exposure	Offspring age of testing	Drug	Paradigms/tests used	Summary of relevant findings	Sex differences
(Eriksson & Rönnbäck, 1989)	Sprague-Dawley rat	Liquid diet orally	Four days pre-conception – GD 21	Adult	Dams: 12.5, 25, 50, 100, or 150 mg/kg/day oral MOR. Offspring hot-plate testing: MOR (7.5 mg/kg, i.p.)	Neonatal survival rate & BW, hot-plate thermal hyperalgesia test	↑ MOR intake leads to ↓ pup survival rate. ↓ BW (PND 0-19). ↓ response latency to 7.5 mg/kg MOR in hot-plate test.	Only ♂ offspring used
(L. Y. Wu et al., 2009)	Sprague Dawley rat	s.c. injection 2x/day	8 days preconception- PND 0	Adult	Dams: 2 – 6 mg/kg MOR. HCl (with or without dextrom) Offspring CPP & locomotor test: 1 mg/kg MOR	CPP, locomotor activity test, HPLC for dopamine and serotonin metabolites in NAc	CPP & ↑ locomotion (sensitization) after low-dose MOR. ↑ dopamine & 5-HT turnover rate in NAc. All reversed by gestational dextrom.	Only ♂ offspring used

(Timár et al., 2010)	Wistar rat	s.c. injection	Experiment 1: GD 1 – lactation; Experiment 2: only gestation or only lactation	Neonate & adolescent testing	Dams: 5 mg/kg – 10 mg/kg MOR Offspring for CPP: 1 mg/kg or 3 mg/kg MOR	BW, locomotor activity, tail-flick test, MOR CPP	↓ BW at PND 1, ↑ BW at PND 14 & 21. ♂ MOR offspring had ↑ locomotion at PND 23 (not ♀). No change in tail-flick test at PND 24. MOR-induced CPP seen for ♂ & ♀ MOR-offspring.	Both sexes examined; Yes sex differences

Appendix Table 1 Abbreviations: MOR: morphine, **GD**: gestation day, **BW** = body weight, **s.c.**: sub-cutaneous, **dextrom** = dextromethorphan, **CPP**: conditioned place preference, **HPLC**: high performance liquid chromatography, **NAc** = nucleus accumbens, **i.p.**: intraperitoneally, PND = postnatal day; **HCl**: hydrochloride; **5-HT**: serotonin

Appendix Table 2. Most commonly cited preclinical studies in our review investigating the effects of <u>partial gestational</u> morphine on offspring outcomes.

Reference	**Animal model**	**Parental route of administration**	**Offspring age of exposure**	**Offspring age of testing**	**Drug**	**Paradigms/tests used**	**Summary of relevant findings**	**Sex differences**
(Jóhannesson & Becker, 1972)	SpragueDawley rat	s.c. injection	Four groups: GD 2-5, GD 7-9, GD 11-13, GD 17-20	GD 22 for teratology experiment; PND 12-3, PND 21-22 for hot-plate test	Dams *in utero*: 20 mg/kg MOR sulfate Dam challenge: 10 mg/kg MOR Offspring hot-plate test: 0.45 mg/kg & 0.625 mg/kg	Teratology, hot-plate test after MOR challenge	Neonate BW depends on dam gestation MOR length. ↓ reaction time in hot-plate test in MOR dams & PND 12-13 MOR offspring challenged with MOR.	None observed

					MOR			
(Wagner et al., 1986)	Sprague Dawley rat	s.c injection 2x/day	GD 5-12	Adult	Dams: 5 mg/kg-10 mg/kg MOR sulfate Offspring SA: 2- 16 mg/kg MOR or 0.5-4 mg/kg AMPHET	Fixed-interval water SA following MOR or AMPHET injection	MOR-offspring display tolerance after acute MOR. Similar response rate after acute AMPHET injection. No difference in central catecholamines.	Both sexes used; Yes, sex differences
(De Vries et al., 1991)	Wistar rat	Semi-synthetic diet	14 days during gestation	GD 21 fetus	0.5 mg/kg - 1 mg/kg MOR	Adenylate cyclase activity, neurotransmitter release in striatal slices	↑ D1 stimulated cAMP release & ↑ NA release by DAGO. ↑ cAMP after D1R agonist stimulation. ↑ % NA release after calcium stimulation.	Sex used not specified
(Ramsey et al., 1993)	Wistar rat	s.c. injection 2x/day (GD 7-9), followed by oral solution	GD 7-PND 0	CF on PND 1, neonatal & adult testing	Dams: 10 mg/kg MOR HCl injected GD 7- 9, 0.5 mg/mL MOR HCl in 0.5% sucrose water Offspring: cocaine HCl (0.125 mg/kg per infusion), heroin HCl (0.063 mg/kg per infusion)	Neonatal outcomes (BW, sex ratio, survival rate), I.V. SA of cocaine & heroin	MOR dams had ↓ fluid consumption. No difference in neonatal outcomes. ↑ infusions of heroin or cocaine in adult MOR-offspring	Only ♂ offspring used
(Gagin et al., 1997)	Fischer rat	s.c. injection	GD 12 – 18	CF on PND 1, testing as adults	Dams: increasing concentration of slow-release	Maternal food intake, neonatal outcomes (BW, litter size, etc),	↓ food intake for MOR dams; no differences in pup BW;	Both sexes used, No sex differences

					emulsion MOR sulfate from 0.75-12.0 mg/injection. Offspring CPP: 2.0 mg/kg MOR HCl	CPP	↑ in CPP MOR preference score in MOR offspring	reported
(Dutriez-Casteloot et al., 1999)	Wistar rats	s.c. injection 2x/day	GD 11 - 18	After weaning (PND 21)	10 mg/kg/day MOR	RIA for CRF, ACTH, CORT, LH and testosterone levels; HPLC to determine catecholamine (NA, A) levels; weighing of adrenal gland, testicle and seminal vesicle	No difference in BW, testicle weight, seminal vesicle weight, LH levels, testosterone levels, MRs & GRs in hypothalamus & hippocampus. ↓ levels of adrenal NA & A but ↑ in plasma A	Only ♂ offspring used
(Jean Lesage et al., 2000)	Wistar rat	s.c. injection 2x/day	GD 11 - 18	PND 0	10 mg/kg MOR sulfate	NADPH histochemistry in adrenals, Radioimmunoassay	↓ adrenal weight, ↑ adrenal aldosterone content & plasma aldosterone levels. No difference in Na^+/K^+ ratio. ↓ aldosterone release to angiotensin II & ACTH. ↑ aldosterone release after high K^+ stimulation.	Both sexes used; No sex difference so data pooled
(Šlamberová et al., 2001)	Sprague-Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Symmetrical maze, eight-arm radial maze	Faster completion of visual & nonvisual trials. ↑ time to complete	Both sexes used; Yes sex differe

							radial arm maze trials. ↑ memory errors in ♀ MOR offspring (dependent on gonadal hormones).	nces
(Šlamberová et al., 2002)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Gonadal hormonal replacement, Effects of cold-water stressor on FST and OFA	Male and OVX-female offspring from saline & MOR dams show ↑ depressive-like behavior in FST. ↓ locomotor activity in OFA & ↑ response to cold water stressor in ♂ MOR-offspring.	Both sexes; yes, sex differences
(Rimanóczy et al., 2003)	Sprague-Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Blood sample collection, RIA for CORT and ACTH, restraint stress, DST	↓ levels of ACTH after restraint stress. Basal CORT or ACTH unchanged. No difference in increased CORT/ACTH after DST	Only ♂ offspring used
(Šlamberová, Rimanóczy, et al., 2003)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Gonadal hormonal replacement, DAMGO binding using autoradiography	No difference in μ-opioid receptor density in hippocampus of ♂ MOR offspring. ↑ CA1 & ↓ CA3 μ-opioid receptor density of MOR OVX ♀ offspring, dependent on estrogen &/or progesteron	Both sexes used; Yes sex difference

							e.	
(Ilona Vathy et al., 2003)	Sprague-Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Treatment with sex hormones, μ -opioid receptor autoradiography	Gonadally-intact ♂ (not ♀) MOR offspring had ↑ receptor binding density in NAc, PMCoA and CeA, but ↓ in BLA and LA. No effect in BNST.	Both sexes used; Yes sex differences
(Velíšek et al., 2003)	Sprague Dawley rat	s.c injections 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Synaptic transmission in LPP	Prenatal MOR ↓ baseline EPSP and synaptic plasticity in the LPP.	Only ♂ offspring used
(Schindler et al., 2004)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	ISH, Northern blot, radioimmunoassay, receptor autoradiography for hippocampal opioid mRNA, peptides, & receptors	Unchanged proenkephalin or prodynorphin mRNA in DG in northern blot. ↓ proenkephalin & ↑ prodynorphin mRNA levels in MOR-offspring. using ISH. ↑ DAMGO binding in hippocampus. ↑ latency to seizure onset. Reversed with naloxone.	Only ♂ offspring used
(Šlamberová et al., 2004)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Blood sample collection, RIA for CORT and ACTH, restraint stress, DST	Unchanged basal CORT & ACTH. ↓ stress-induced ACTH. Unchanged stress-induced CORT. No	Only ♀ offspring used

							difference in DST-induced increase in ACTH. No DEX-induced ↓ in CORT levels at lower DEX doses.	
(Che et al., 2005)	Lohmann brown eggs	Daily injection into airspace of eggs	Embryonic Day 12-16	24-hours after hatching	20 mg/kg; MOR HCl	Hatch and egg weight; one-trial passive avoidance learning task	No difference in egg or hatch weight. ↓ hatch day. Unchanged avoidance ratio for STM. ↓ mean avoidance ratio for LTM.	Sex used not specified
(Laborie et al., 2005)	Wistar rat	s.c. injection 2x/day	GD 11 - 18	Adult	10 mg/kg MOR sulfate	3-min. ether inhalation stress procedure, Radioimmunoassay, HPLC to measure 5-HT & 5HIAA levels in hippocampus & hypothalamus	No difference in body growth, plasma ACTH & CORT at baseline or after ether stress. ↓ adrenal baseline NE & 90 mins. after ether stress. ↓ PNMT mRNA at 60 mins. & 120 mins. after ether stress. ↑ basal & stress-induced HT & 5HIAA in hypothalamus.	Only ♂ offspring used (mentioned in discussion section only)
(Riley & Vathy, 2006)	Sprague-Dawley rat	s.c. injection 2x/day	GD 11-18	CF on PND 1, adult testing	Dams: 5 mg/kg – 10 mg/kg MOR sulfate Offspring CPP: 0.1, 0.3, 1, 3, 5 mg/kg	CPP, MOR and food SA	Unchanged MOR CPP & food SA. ↑ lever presses (1 mg/kg MOR). Controls had ↑ lever presses during	Only ♂ offspring used

					MOR sulfate		extinction.	
(Rimanóczy et al., 2006)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Adrenalectomy, MR & GR binding assay	MR & GR B_{max} in ♂ MOR- offspring same as control offspring (not saline offspring) in hippocampus. Unchanged MR & GR B_{max} in hypothalamus. ↑ GR B_{max} & ↓ MR B_{max} in hippocampus of all estrus ♀ offspring.	Both sexes; yes, sex differences
(I. Vathy et al., 2007)	Sprague Dawley rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, adult testing	5 mg/kg – 10 mg/kg MOR sulfate	Cocaine & food lever pressing operant conditioning	No difference in cocaine SA FR1. ↑ cocaine active lever pressing in CF rats. ↓ cocaine SA in proestrus cycle ♀ rats.	Both sexes used; Yes sex difference
(Chiang et al., 2010)	Sprague Dawley rat	s.c. injection 2x/day	GD 3 - 20	Adult	Dams: Group 1: 2 - 4 mg/kg MOR MOR offspring tail-flick test: 10 mg/kg MOR	Neonatal outcomes (# of offspring, % mortality, BW), tail-flick test	↑ BW on PND 7. ↓ latency in tail-flick test after MOR injection.	Only ♂ offspring used
(He et al., 2010)	fertilized "BAU-3" eggs	Intermittent (every other day) injection into eggs	Four groups: Embryonic Day 5–8, 9–12, 13–16 and 17–20	PND 1-7	1 mg/kg MOR Offspring for. CPP: 1 mg/kg	One-trial passive avoidance learning task; MOR CPP	↓ % avoidance in E5-8 MOR- offspring. ↓ % avoidance at 360 mins. post-training in E9-12 and E17-20 MOR	Sex used not specified

					MOR, i.p.		offspring. E9-12 and E17-20 MOR- offspring display MOR-induced CPP. MOR-CPP 72 hrs post-conditioning in E17-20 MOR- offspring.	
(J. Jiang et al., 2011)	fertilized "BAU-3" eggs	Intermittent (every other day) injection into eggs	Embryonic Day 5–8	PND 1-7	1 mg/kg MOR Offspring for. CPP: 1 mg/kg MOR, i.p.	One-trial passive avoidance learning task; MOR CPP; electrophysiology in IMM	↓ % avoidance at 10 mins., 360 mins., & 720 mins. No MOR-induced CPP. ↓ % paired-pulse facilitation, paired-pulse ratio, & % induction rate of LTP.	Sex used not specified
(Nasiraei-Moghadam et al., 2013)	Wistar rat	Oral solution	Two groups: GD 1-13, GD 1-PND 7	PND 28 & PND 70	0.01 – 0.08 mg/mL MOR sulfate in water	Electric shock passive avoidance training & testing, Western blotting for CAMKII, BDNF, cleaved caspase-3, & NT-3 protein, IHC for Bax & Bcl-2	Unchanged passive avoidance acquisition. ↓ step through latency in ♀ adolescents & adults (depends on *in utero* MOR). ↑ Bax & ↓ Bcl-2 IHC in ♀ adolescents & adults. ↑ caspase-3 & ↓ BDNF protein levels in ♀ adolescents & adults.	Both sexes used; Yes sex difference

(Chiang et al., 2014)	Sprague Dawley rat	s.c. injection 2x/day	GD 3 - 20	Adult	Group 1: 2 - 4 mg/kg MOR, Group 2: 5 - 7 mg/kg METH; Group 3: BUP 3 mg/kg once a day	Locomotor activity test, METH CPP, RT-PCR, Adenylyl cyclase activity assay	↑ METH-induced locomotion (sensitization) & CPP for low-dose METH seen in BUP-offspring. ↓ Drd1a/GAPDH mRNA ratio & ↓ cAMP levels in NAc of BUP-offspring. D1R receptor agonist ↑ cAMP levels in NAc in BUP-offspring. Unchanged in MOR- or methadone offspring.	Only ♂ offspring used
(A. Ahmadalipour et al., 2015)	Wistar rat	s.c injections 2x/day	GD 11 - 18	CF on PND 1, late adolescent - adult testing	5 mg/kg – 10 mg/kg MOR sulfate	EE given from adolescence-adulthood, LDB, EPM, Passive avoidance test, FST, ELISA for hippocampal BDNF	↑ anxiety-like behavior in LDB & EPM. ↓ step-though latency in passive avoidance memory test. ↑ swim time in FST. ↓ BDNF levels. All effects improved by EE.	Both sexes; yes, sex differences

(Tan et al., 2015)	Sprague Dawley rat	s.c. injection 2x/day	GD 9 - 18	Adult	5 mg/kg – 10 mg/kg MOR HCl	Foot shock contextual fear conditioning & extinction, foot shock pain threshold test, open-field test, EPM, LDB test, electrophysiology in hippocampus	↑ % freezing during extinction. ↓ anxiety-like behavior in EPM & LDB. Unchanged locomotion. Impaired hippocampal LTP & LTD (fEPSP) at baseline & after fear conditioning. Unchanged fEPSP & baseline amplitude.	Only ♂ offspring used
(Shen et al., 2016)	Sprague Dawley rat	s.c. injection 2x/day	GD 3 - 20	Adulthood	2 - 4 mg/kg MOR HCl	METH CPP, METH I.V. self-administration, food SA	Unchanged neonatal outcomes & acquisition METH CPP. CPP extinction was delayed. Enhanced METH-primed reinstatement CPP. Unchanged FR1 and PR. ↑ METH-primed SA reinstatement. No difference in food SA.	Sex used not specified
(Y. Wang, Yao, Li, et al., 2017)	fertilized "BAU-3" eggs	Daily injection into eggs	Embryonic Day 17 - 19	PND 1 - 4	1 mg/kg MOR Offspring for CPP: 1 mg/kg MOR, i.p.	MOR CPP; WB of the PKCα isoform in IMM	Unchanged hatch weight. Longer hatch day & MOR-induced CPP. ↑ locomotion in CPP post-test. ↓ PKCα levels in membrane fraction, but ↑ in cytosol fraction of IMM.	Sex used not specified

							Unchanged basal PKCα in IMM.	
(Y. Wang, Yao, Nie, et al., 2017)	fertilized "BAU-3" eggs	Intermittent (every other day) injection into eggs	Embryonic Day 5 – 8	Second day post-hatch	1 mg/kg MOR	Hatch weight; one-trial passive avoidance learning task; WB of the PKC isoforms in IMM	Unchanged hatch weight. Longer hatch day. ↓ % avoidance at 30, 120, & 360 min.. ↓ PKCα IMM levels in membrane fraction, but ↑ in cytosol fraction. ↑ PKCδ IMM levels in membrane fraction, but ↓ in cytosol fraction.	Sex used not specified
(Ali Ahmadalipour et al., 2018)	Wistar rat	s.c. injection 2x/day	GD 11 - 18	CF on PND 1, testing from PND 21 - 51 ♂ & ♀	5 mg/kg – 10 mg/kg MOR sulfate	MWM, hippocampal LTP recording, ELISA for BDNF protein	↑ latency & swim distance in MWM acquisition, impaired LTP induction and ↓ BDNF in ♀ (reversed by exercise and EE)	Both sexes used; Yes sex differences

Appendix Table 2 Abbreviations: MOR: morphine**, GD**: gestation day**, BW**: body weight**, s.c.**: sub-cutaneous, **SA**: self-administration**, AMPHET**: amphetamine, CF: cross-fostered**, RT-PCR**: Real-Time Polymerase Chain Reaction**, METH**:

methamphetamine**, CPP**: conditioned place preference**, MWM**: Morris Water Maze**,** LTP: long-term potentiation**, BDNF**: brain-derived neurotrophic factor, **EE**: enriched environment**, EPM**: Elevated Plus Maze**, FST**: Forced Swim Test**, RIA**: radioimmunoassay, **ACTH**: adreno-corticotropin releasing hormone**, CORT**: corticosterone**, DST**: dexamethasone suppression test**, LH**: luteinizing hormone**, HPLC**: high performance liquid chromatography**, NA**: noradrenaline**, A**: adrenaline**, MR**: mineralocorticoid receptors**, GR**: glucocorticoid receptors**, i.p.**: intraperitoneally, **FR1**: fixed ratio 1, **PR**: progressive ratio**, BUP**: buprenorphine**, Drd1a/D1R**: dopamine D1 receptor**, LDB**: light-dark box**, fEPSP**: field excitatory postsynaptic potential**, IHC**: immunohistochemistry**, DAMGO**: [D-Ala2, MePhe4, Gly-ol5]enkephalin, **DG**: dentate gyrus**, ISH**: *In situ* hybridization**, LPP**: lateral perforant path**, OFA**: open field arena**, 5-HT**: serotonin, **5HIAA**: 5-hydroxylindoleacetic acid**, PNMT**: phenylethanolamine N-methyltransferase, **OVX, EB**: ovariectomized, treated with estradiol**, IMM**: intermediate medial mesopallium, **PND**: postnatal day, **I.V.**: intravenous; **HCl**: hydrochloride, **cAMP**: Cyclic Adenosine Monophosphate, DAGO: [D-Ala2,MePhe4,Gly-ol5]enkephalin, **CRF**: corticotropin-releasing factor, **BLA**: basolateral amygdala, **BNST**: bed nucleus of stria terminalis, **LA**: lateral amygdala, **RNA**: ribonucleic acid, **STM**: short-term memory, **LTM**: long-term memory, **DEX**: dexamethasone, **NE**: norepinephrine, **Bmax**: maximal binding capacity, **GAPDH**: Glyceraldehyde 3-phosphate dehydrogenase, **NAc**: nucleus accumbens, **ELISA**: enzyme-linked immunoassay, **LTD**: long-term depression, **WB**: western blot, **PKC**: protein kinase C, **PMCoA**: posteromedial cortical amygdaloid nuclei, **E**: embryonic day, **Bax**: BCL2-Associated X Protein, **Bcl-2**: B-cell lymphoma 2

Appendix Table 3. Most commonly cited preclinical studies in our review investigating the effects of gestation+lactation morphine exposure on offspring outcomes.

Reference	Animal model	Parental route of administration	Offspring age of exposure	Offspring age of testing	Drug	Paradigms/tests used	Summary of relevant findings	Sex differences
(Glick et al., 1977a)	Sprague-Dawley rat	Oral solution	GD 7 - lactation	PND 90	Dams: 0.5 mg/mL MOR sulfate in 0.4 mg/mL saccharin Offspring: 10 µL infusion of 0.01 mg MOR sulfate per	MOR I.V. SA	MOR offspring reached SA criterion faster	Only ♀ offspring used

					lever press			
(Siddiqui et al., 1997)	Wistar rat	i.p. injection	40 days preconception, throughout gestation - PND 10	Adult	5–40 mg/kg MOR sulfate	Radioimmunoassay for hormone levels, ♀ reproductive behavior & solicitation behavior with stimulus ♂, catecholamine levels measured using HPLC	↓ pup BW, ↓ ovarian weights. ↓ ovarian estradiol and progesterone, and ↓ plasma luteinizing-hormone and estradiol levels. ↓ lordosis behavior. ↓ NE in hypothalamus	Only ♀ offspring used
(Chiou et al., 2003)	Sprague-Dawley rat	s.c. injection	Seven days pre-conception – lactation	PND 14	MOR dams: 2 mg/kg then ↑ 1 mg/kg per week; ↑ 1 mg/kg every 2 weeks (PND 0 - 30). Offspring tail-flick test: 2 µg/2 µL MOR	Tail-flick test, µ-opioid receptor autoradiography, brain slice preparations, patch-clamp recordings	↓ antinocicepti on. µ-opioid receptor density ↓ in striatum, thalamus, amygdala. No change in K^+ channel activation or membrane potential properties in ventrolateral PAG neurons	None observed
(Yang et al., 2003b)	Sprague-Dawley rat	s.c. injection 2x/day	Seven days pre-conception – lactation	PND 14 - 31	MOR HCl starting at 2 mg/kg but ↑ 1 mg/kg per week; ↑ by 1 mg/kg every 2 weeks (PND 0-30)	MWM, hippocampal slice preparation, whole-cell recordings of CA1 pyramidal cells	No difference in LTP after HFS on PND 14. ↓ LTD. ↑ escape latency in MWM acquisition on PND 31. ↓ pCREB in hippocampal CA1.	Sex used not specified

(Sobor et al., 2010)	Wistar rat	s.c. injection	GD 1 - lactation	Maternal behavior tested from offspring PND 2 - 9.	Dams: 5 mg/kg – 10 mg/kg MOR HCl Dam challenge: 1, 3, 10 or 20 mg/kg MOR HCl	Maternal behavior observation, retrieval latency test, tail flick test after MOR challenge, neonate outcomes	↓ BW on PND 0. Unchanged neonate litter size, sex ratio, MOR BW. ↓ dam maternal behavior. ↑ in non-maternal behavior & ↑ in latency to retrieve 1st pup of the litter after MOR challenge	Only ♀ dams tested
(Timár et al., 2010)	Wistar rat	s.c. injection	Experiment 1: GD 1 – lactation; Experiment 2: only gestation or only lactation	Neonate & adolescent testing	Dams: 5 mg/kg – 10 mg/kg MOR Offspring for CPP: 1 mg/kg or 3 mg/kg MOR	BW, locomotor activity, antinociception through tail-flick test, MOR CPP	↓ BW on PND 1, ↑ BW on PND 14 & 21. ♂ MOR offspring had ↑ locomotion on PND 23 (not ♀). No difference in tail-flick test on PND 24. MOR-induced CPP seen for ♂ & ♀.	Both sexes used; Yes sex differences
(Klausz et al., 2011)	Wistar rat	s.c. injection	GD 1 - lactation	Juvenile & adult testing	5 mg/kg – 10 mg/kg MOR HCl	Resting blood glucose measurement & thymus/spleen/adrenal gland weights, EPM, FST, RIA to measure plasma ACTH & plasma CORT	↓ BW & weight in all glands. ↓ basal ACTH in adolescents& adults. ↓ basal CORT in adolescents, but ↑ in adults. Trend for ↓ ACTH after EPM and FST. Unchanged CORT after EPM, but ↓ CORT after FST. Unchanged in EPM or FST.	Only ♂ offspring used

(Nasiraei-Moghadam et al., 2013)	Wistar rat	Oral solution	Two groups: GD 1-13, GD 1- PND 7	PND 28 & PND 70	0.01 – 0.08 mg/mL MOR sulfate in water	Electric shock passive avoidance training and testing, Western blotting for CAMKII, BDNF, cleaved caspase-3, & NT-3 protein, IHC for Bax & Bcl-2	No difference in passive avoidance acquisition. ↓ step through latency in ♀ adolescents & adults (depends on *in utero* MOR). ↑ Bax & ↓ Bcl-2 IHC in ♀ adolescents & adults. ↑ caspase-3 & ↓ BDNF protein levels in ♀ adolescents & adults.	Both sexes used; Yes sex difference
(P. L. Wu et al., 2018)	Sprague Dawley rat	s.c. injection 2x/day	7 days preconception - PND 30	PND 14 & PND 30	MOR HCl starting at 2 mg/kg but ↑ 1 mg/kg every week; ↑ by 1 mg/kg every 2 weeks (PND 0 - 30)	mRNA levels using qPCR	↓ NMDA & AMPA receptor subunits. PSD-95 deficits in NAc, VTA, PFC at PND 14 & PND 30	Sex used not specified

Appendix Table 3 Abbreviations: MOR: morphine**, GD**: gestation day**, BW**: body weight**, s.c.**: sub-cutaneous, **SA**: self-administration**, PSD-95**: Postsynaptic density protein**, PCR**: Polymerase Chain Reaction**, AMPA**: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**, NMDA**: *N*-methyl-d-aspartate, **CPP**: conditioned place preference**, MWM**: Morris Water Maze**, LTP**: long-term potentiation**, BDNF**: brain-derived neurotrophic factor, **EPM**: Elevated Plus Maze**, FST**: Forced Swim Test**, RIA**: radioimmunoassay, **ACTH**: adreno-corticotropin releasing hormone**, CORT**: corticosterone**, HPLC**: high performance liquid chromatography**, NA**: noradrenaline**, A**: adrenaline**, NE**: norepinephrine**, i.p.**: intraperitoneally, **pCREB**: phosphorylated cAMP response element-binding protein**, IHC**: immunohistochemistry**, PND**: postnatal day, **I.V.**: intravenous; **HCl**: hydrochloride, **RNA**: ribonucleic acid, **NAc**: nucleus accumbens, **LTD**: long-term depression, **Bax**: BCL2-Associated X Protein, **Bcl-2**: B-cell lymphoma 2, **VTA**: ventral tegmental area, **PFC**: prefrontal cortex, **PAG**: periaqueductal gray, **HFS**: high-frequency stimulation, **CAMKII**: calmodulin-dependent protein kinase II

BIBLIOGRAPHY

Abraham, A. D., Casello, S. M., Schattauer, S. S., Wong, B. A., Mizuno, G. O., Mahe, K., Tian, L., Land, B. B., & Chavkin, C. (2021). Release of endogenous dynorphin opioids in the prefrontal cortex disrupts cognition. *Neuropsychopharmacology*, *46*(13), 2330–2339. https://doi.org/10.1038/s41386-021-01168-2

Adinoff, B. (2004). Neurobiologic processes in drug reward and addiction. In *Harvard Review of Psychiatry* (Vol. 12, Issue 6, pp. 305–320). https://doi.org/10.1080/10673220490910844

Afful, S. E., Strickland, J. R., Cottler, L., & Bierut, L. J. (2010). Exposure to trauma: A comparison of cocaine-dependent cases and a community-matched sample. *Drug and Alcohol Dependence*, *112*(1–2), 46–53. https://doi.org/10.1016/j.drugalcdep.2010.05.012

Ahmadalipour, A., Ghodrati-Jaldbakhan, S., Samaei, S. A., & Rashidy-Pour, A. (2018). Deleterious effects of prenatal exposure to morphine on the spatial learning and hippocampal BDNF and long-term potentiation in juvenile rats: Beneficial influences of postnatal treadmill exercise and enriched environment. *Neurobiology of Learning and Memory*, *147*, 54–64. https://doi.org/10.1016/j.nlm.2017.11.013

Ahmadalipour, A., Sadeghzadeh, J., Vafaei, A. A., Bandegi, A. R., Mohammadkhani, R., & Rashidy-Pour, A. (2015). Effects of environmental enrichment on behavioral deficits and alterations in hippocampal BDNF induced by prenatal exposure to morphine in juvenile rats. *Neuroscience*, *305*, 372–383. https://doi.org/10.1016/j.neuroscience.2015.08.015

Ajdary, M., Farzan, S., Razavi, Y., Arabdolatabadi, A., & Haghparast, A. (2021). Effects of morphine on serum reproductive hormone levels and the expression of genes involved in fertility-related pathways in male rats. *Iranian Journal of Pharmaceutical Research*, *20*(1), 153–164. https://doi.org/10.22037/ijpr.2019.112119.13544

Akbarabadi, A., Niknamfar, S., Vousooghi, N., Sadat-Shirazi, M. S., Toolee, H., & Zarrindast, M. R. (2018). Effect of rat parental morphine exposure on passive avoidance memory and morphine conditioned place preference in male offspring. *Physiology and Behavior*, *184*, 143–149. https://doi.org/10.1016/j.physbeh.2017.11.024

Al-Hasani, R., & Bruchas, M. R. (2011). Molecular mechanisms of opioid receptor-dependent signaling and behavior. In *Anesthesiology* (Vol. 115, Issue 6, pp. 1363–1381). NIH Public Access. https://doi.org/10.1097/ALN.0b013e318238bba6

Andersen, J. M., Høiseth, G., & Nygaard, E. (2020). Prenatal exposure to methadone or buprenorphine and long-term outcomes: A meta-analysis. In *Early Human Development* (Vol. 143, p. 104997). Elsevier Ireland Ltd. https://doi.org/10.1016/j.earlhumdev.2020.104997

Arias, A. J., & Kranzler, H. R. (2008). Treatment of co-occurring alcohol and other drug use disorders. In *Alcohol Research and Health* (Vol. 31, Issue 2, pp. 155–167). National Institute on Alcohol Abuse and Alcoholism.

Arnaudo, C. L., Andraka-Christou, B., & Allgood, K. (2017). Psychiatric Co-Morbidities in Pregnant Women with Opioid Use Disorders: Prevalence, Impact, and Implications for Treatment. In *Current Addiction Reports* (Vol. 4, Issue 1, p. 1). Springer. https://doi.org/10.1007/s40429-017-0132-4

Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J. H., Nakano, A. H., Dado, R. J., Loh, H. H., Law, P. Y., Wessendorf, M. W., & Elde, R. (1995). Distribution and targeting of a μ-opioid receptor (MOR1) in brain and spinal cord. *Journal of Neuroscience*, *15*(5 I), 3328–3341. https://doi.org/10.1523/jneurosci.15-05-03328.1995

Avishai-Eliner, S., Brunson, K. L., Sandman, C. A., & Baram, T. Z. (2002). Stressed-out, or in (utero)? In *Trends in Neurosciences* (Vol. 25, Issue 10, pp. 518–524). Trends Neurosci. https://doi.org/10.1016/S0166-2236(02)02241-5

Azarov, A. V., & Woodward, D. J. (2014). Early ethanol and water consumption: Accumulating experience differentially regulates drinking pattern and bout parameters in male alcohol preferring (P) vs. wistar and sprague dawley rats. *Physiology and Behavior*, *123*, 20–32. https://doi.org/10.1016/j.physbeh.2013.09.015

Azuine, R. E., Ji, Y., Chang, H. Y., Kim, Y., Ji, H., Dibari, J., Hong, X., Wang, G., Singh, G. K., Pearson, C., Zuckerman, B., Surkan, P. J., & Wang, X. (2019). Prenatal Risk Factors and Perinatal and Postnatal Outcomes Associated with Maternal Opioid Exposure in an Urban, Low-Income, Multiethnic US Population. *JAMA Network Open*, *2*(6), e196405–e196405. https://doi.org/10.1001/jamanetworkopen.2019.6405

Bagdas, D., Jackson, A., Carper, M., Chen, R. Y. T., Akinola, L. S., & Damaj, M. I. (2020). Impact of menthol on nicotine intake and preference in mice: Concentration, sex, and age differences. *Neuropharmacology*, *179*, 108274. https://doi.org/10.1016/j.neuropharm.2020.108274

Baharnoori, M., Bhardwaj, S. K., & Srivastava, L. K. (2012). Neonatal behavioral changes in rats with gestational exposure to lipopolysaccharide: A prenatal infection model for developmental neuropsychiatric disorders. *Schizophrenia Bulletin*, *38*(3), 444–456. https://doi.org/10.1093/schbul/sbq098

Bailey, B. A., Shah, D. S., Boynewicz, K. L., Justice, N. A., & Wood, D. L. (2022). Impact of in utero opioid exposure on newborn outcomes: beyond neonatal opioid withdrawal syndrome. *Journal of Maternal-Fetal and Neonatal Medicine*. https://doi.org/10.1080/14767058.2022.2035713

Bailey, K. R., & Crawley, J. N. (2009). Anxiety-Related Behaviors in Mice. In *Methods of Behavior Analysis in Neuroscience*. CRC Press/Taylor & Francis.

Baker, E. J., Walter, N. A. R., Salo, A., Rivas Perea, P., Moore, S., Gonzales, S., & Grant, K. A. (2017). Identifying Future Drinkers: Behavioral Analysis of Monkeys Initiating Drinking to Intoxication is Predictive of Future Drinking Classification. *Alcoholism: Clinical and Experimental Research*, *41*(3), 626–636. https://doi.org/10.1111/acer.13327

Bakhireva, L. N., Holbrook, B. D., Shrestha, S., Leyva, Y., Ashley, M., Cano, S., Lowe, J., Stephen, J. M., & Leeman, L. (2019). Association between prenatal opioid exposure,

neonatal opioid withdrawal syndrome, and neurodevelopmental and behavioral outcomes at 5–8 months of age. *Early Human Development*, *128*, 69–76. https://doi.org/10.1016/j.earlhumdev.2018.10.010

Bali, A., Randhawa, P. K., & Jaggi, A. S. (2015a). Stress and opioids: Role of opioids in modulating stress-related behavior and effect of stress on morphine conditioned place preference. In *Neuroscience and Biobehavioral Reviews* (Vol. 51, pp. 138–150). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2014.12.018

Bali, A., Randhawa, P. K., & Jaggi, A. S. (2015b). Stress and opioids: Role of opioids in modulating stress-related behavior and effect of stress on morphine conditioned place preference. In *Neuroscience and Biobehavioral Reviews* (Vol. 51, pp. 138–150). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2014.12.018

Barker, J. M., Taylor, J. R., De Vries, T. J., & Peters, J. (2015). Brain-derived neurotrophic factor and addiction: Pathological versus therapeutic effects on drug seeking. *Brain Research*, *1628*(0 0), 68–81. https://doi.org/10.1016/j.brainres.2014.10.058

Barr, G. A., McPhie-Lalmansingh, A., Perez, J., & Riley, M. (2011). Changing mechanisms of opiate tolerance and withdrawal during early development: Animal models of the human experience. *ILAR Journal*, *52*(3), 329–341. https://doi.org/10.1093/ilar.52.3.329

Barr, G. A., & Wang, S. (1992). Tolerance and withdrawal to chronic morphine treatment in the week-old rat pup. *European Journal of Pharmacology*, *215*(1), 35–42. https://doi.org/10.1016/0014-2999(92)90605-4

Behnke, M. M., & Smith, V. M. (2013). Prenatal Substance Abuse: Short-and Long-term Effects on the Exposed Fetus. *American Academy of Pediatrics*, *131*(1). https://doi.org/10.1542/peds.2012-3931

Belda, X., Fuentes, S., Labad, J., Nadal, R., & Armario, A. (2020). Acute exposure of rats to a severe stressor alters the circadian pattern of corticosterone and sensitizes to a novel stressor: Relationship to pre-stress individual differences in resting corticosterone levels. *Hormones and Behavior*, *126*, 104865. https://doi.org/10.1016/j.yhbeh.2020.104865

Belknap, J. K. (1990). Physical dependence induced by the voluntary consumption of morphine in inbred mice. *Pharmacology, Biochemistry and Behavior*, *35*(2), 311–315. https://doi.org/10.1016/0091-3057(90)90161-A

Belknap, J. K., Crabbe, J. C., Riggan, J., & O'Toole, L. A. (1993). Voluntary consumption of morphine in 15 inbred mouse strains. *Psychopharmacology*, *112*(2–3), 352–358. https://doi.org/10.1007/BF02244932

Benchorin, G., Calton, M., Beaulieu, M., & Vollrath, D. (2017). Assessment of Murine Retinal Function by Electroretinography. *BIO-PROTOCOL*, *7*(7). https://doi.org/10.21769/bioprotoc.2218

Berrendero, F., Kieffer, B. L., & Maldonado, R. (2002). Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in μ-opioid receptor knock-out mice. *Journal of Neuroscience*, *22*(24), 10935–10940. https://doi.org/10.1523/jneurosci.22-24-10935.2002

Berrettini, W. (2013). Opioid pharmacogenetics of alcohol addiction. *Cold Spring Harbor Perspectives in Medicine*, *3*(7), a012203. https://doi.org/10.1101/cshperspect.a012203

Betcher, H. K., Vande Voort, J. L., Croarkin, P. E., Gandhi, K. D., Shekunov, J., Larrabee, B. R., Limbecka, M. G., Rummans, T. A., & Romanowicz, M. (2019). Outcomes of Children of Parents in Medication-Assisted Treatment Compared to Healthy Controls. *Primary Care Companion to the Journal of Clinical Psychiatry*, *21*(4), 0–0. https://doi.org/10.4088/PCC.19m02474

Bhat, R., Chari, G., & Rao, R. (2006). Effects of prenatal cocaine, morphine, or both on postnatal opioid (μ) receptor development. *Life Sciences*, *78*(13), 1478–1482. https://doi.org/10.1016/j.lfs.2005.07.023

Biederman, J., Faraone, S. V., Monuteaux, M. C., & Feighner, J. A. (2000). Patterns of alcohol and drug use in adolescents can be predicted by parental substance use disorders. *Pediatrics*, *106*(4 I), 792–797. https://doi.org/10.1542/peds.106.4.792

Birnie, M. T., Kooiker, C. L., Short, A. K., Bolton, J. L., Chen, Y., & Baram, T. Z. (2020). Plasticity of the Reward Circuitry After Early-Life Adversity: Mechanisms and Significance. In *Biological Psychiatry* (Vol. 87, Issue 10, pp. 875–884). Elsevier USA. https://doi.org/10.1016/j.biopsych.2019.12.018

Bjork, J. M., Straub, L. K., Provost, R. G., & Neale, M. C. (2017). The ABCD Study of Neurodevelopment: Identifying Neurocircuit Targets for Prevention and Treatment of Adolescent Substance Abuse. In *Current Treatment Options in Psychiatry* (Vol. 4, Issue 2, pp. 196–209). Springer. https://doi.org/10.1007/s40501-017-0108-y

Blaine, S. K., Milivojevic, V., Fox, H., & Sinha, R. (2016). Alcohol effects on stress pathways: Impact on craving and relapse risk. In *Canadian Journal of Psychiatry* (Vol. 61, Issue 3, pp. 145–153). SAGE Publications Inc. https://doi.org/10.1177/0706743716632512

Blednov, Y. A., Walker, D., Martinez, M., & Harris, R. A. (2006). Reduced alcohol consumption in mice lacking preprodynorphin. *Alcohol*, *40*(2), 73–86. https://doi.org/10.1016/j.alcohol.2006.12.002

Blednov, Y. A., Walker, D., Martinez, M., Levine, M., Damak, S., & Margolskee, R. F. (2008). Perception of sweet taste is important for voluntary alcohol consumption in mice. *Genes, Brain and Behavior*, *7*(1), 1–13. https://doi.org/10.1111/j.1601-183X.2007.00309.x

Blumberg, M. S., Sokoloff, G., Tiriac, A., & Del Rio-Bermudez, C. (2015). A valuable and promising method for recording brain activity in behaving newborn rodents. *Developmental Psychobiology*, *57*(4), 506–517. https://doi.org/10.1002/dev.21305

Borrelli, K. N., Yao, E. J., Yen, W. W., Phadke, R. A., Ruan, Q. T., Chen, M. M., Kelliher, J. C., Langan, C. R., Scotellaro, J. L., Babbs, R. K., Beierle, J. C., Logan, R. W., Johnson, W. E., Wachman, E. M., Cruz-Martín, A., & Bryant, C. D. (2021). Sex differences in behavioral and brainstem transcriptomic neuroadaptations following neonatal opioid exposure in outbred mice. *Eneuro*, *8*(5), ENEURO.0143-21.2021. https://doi.org/10.1523/eneuro.0143-21.2021

Bravo, I. M., Luster, B. R., Flanigan, M. E., Perez, P. J., Cogan, E. S., Schmidt, K. T., & McElligott, Z. A. (2020). Divergent behavioral responses in protracted opioid withdrawal in male and female C57BL/6J mice. *European Journal of Neuroscience*, *51*(3), 742–754. https://doi.org/10.1111/ejn.14580

Brennan, D., Wu, T., & Fan, J. (2021). Morphometrical Brain Markers of Sex Difference. *Cerebral Cortex*, *31*(8), 3641–3649. https://doi.org/10.1093/cercor/bhab037

Brieant, A. E., Sisk, L. M., & Gee, D. G. (2021). Associations among negative life events, changes in cortico-limbic connectivity, and psychopathology in the ABCD Study. *Developmental Cognitive Neuroscience*, *52*. https://doi.org/10.1016/j.dcn.2021.101022

Brook, K., Bennett, J., & Desai, S. P. (2017). The Chemical History of Morphine: An 8000-year Journey, from Resin to de-novo Synthesis. *Journal of Anesthesia History*, *3*(2), 50–55. https://doi.org/10.1016/j.janh.2017.02.001

Broussard, C. S., Rasmussen, S. A., Reefhuis, J., Friedman, J. M., Jann, M. W., Riehle-Colarusso, T., & Honein, M. A. (2011). Maternal treatment with opioid analgesics and risk for birth defects. *American Journal of Obstetrics and Gynecology*, *204*(4), 314.e1-314.e11. https://doi.org/10.1016/j.ajog.2010.12.039

Brudzynski, S. (2015). Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate. *Current Neuropharmacology*, *13*(2), 180–192. https://doi.org/10.2174/1570159x13999150210141444

Buckman, S. G., Hodgson, S. R., Hofford, R. S., & Eitan, S. (2009). Increased elevated plus maze open-arm time in mice during spontaneous morphine withdrawal. *Behavioural Brain Research*, *197*(2), 454–456. https://doi.org/10.1016/j.bbr.2008.09.035

Bueno-Junior, L. S., Ruggiero, R. N., Rossignoli, M. T., Del Bel, E. A., Leite, J. P., & Uchitel, O. D. (2017). Acetazolamide potentiates the afferent drive to prefrontal cortex in vivo. *Physiological Reports*, *5*(1). https://doi.org/10.14814/phy2.13066

Burke, A. R., & Miczek, K. A. (2014). Stress in adolescence and drugs of abuse in rodent models: Role of dopamine, CRF, and HPA axis. In *Psychopharmacology* (Vol. 231, Issue 8, pp. 1557–1580). Springer Verlag. https://doi.org/10.1007/s00213-013-3369-1

Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G., & Smith, J. M. (2012). Health Evaluation of Experimental Laboratory Mice. *Current Protocols in Mouse Biology*, *2*(2), 145–165. https://doi.org/10.1002/9780470942390.mo110217

Byrnes, E. M., & Vassoler, F. M. (2018). Modeling prenatal opioid exposure in animals: Current findings and future directions. In *Frontiers in Neuroendocrinology* (Vol. 51, pp. 1–13). Academic Press Inc. https://doi.org/10.1016/j.yfrne.2017.09.001

Calamandrei, G., Valanzano, A., & Alleva, E. (1991). NGF and cholinergic control of behavior: anticipation and enhancement of scopolamine effects in neonatal mice. *Developmental Brain Research*, *61*(2), 237–241. https://doi.org/10.1016/0165-3806(91)90136-7

Campos, A. C., Fogaça, M. V., Aguiar, D. C., & Guimarães, F. S. (2013). Animal models of anxiety disorders and stress. *Revista Brasileira de Psiquiatria*, *35*(SUPPL.2), S101–S111. https://doi.org/10.1590/1516-4446-2013-1139

Cardenas-Iniguez, C., Moore, T. M., Kaczkurkin, A. N., Meyer, F. A. C., Satterthwaite, T. D., Fair, D. A., White, T., Blok, E., Applegate, B., Thompson, L. M., Rosenberg, M. D., Hedeker, D., Berman, M. G., & Lahey, B. B. (2022). Direct and Indirect Associations of Widespread Individual Differences in Brain White Matter Microstructure With Executive Functioning and General and Specific Dimensions of Psychopathology in Children. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, *7*(4), 362–375. https://doi.org/10.1016/j.bpsc.2020.11.007

Carnicella, S., Ron, D., & Barak, S. (2014). Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. In *Alcohol* (Vol. 48, Issue 3, pp. 243–252). Elsevier Inc. https://doi.org/10.1016/j.alcohol.2014.01.006

Carpenter, R. W., Treloar Padovano, H., Emery, N. N., & Miranda, R. (2019). Rate of alcohol consumption in the daily life of adolescents and emerging adults. *Psychopharmacology*, *236*(11), 3111–3124. https://doi.org/10.1007/s00213-019-05262-8

Carroll, M. E., & Anker, J. J. (2010). Sex differences and ovarian hormones in animal models of drug dependence. In *Hormones and Behavior* (Vol. 58, Issue 1, pp. 44–56). Academic Press. https://doi.org/10.1016/j.yhbeh.2009.10.001

Carroll, M. E., Morgan, A. D., Anker, J. J., Perry, J. L., & Dess, N. K. (2008). Selective breeding for differential saccharin intake as an animal model of drug abuse. In *Behavioural Pharmacology* (Vol. 19, Issues 5–6, pp. 435–460). Behav Pharmacol. https://doi.org/10.1097/FBP.0b013e32830c3632

Carroll, M. E., Morgan, A. D., Lynch, W. J., Campbell, U. C., & Dess, N. K. (2002). Intravenous cocaine and heroin self-administration in rats selectively bred for differential saccharin intake: Phenotype and sex differences. *Psychopharmacology*, *161*(3), 304–313. https://doi.org/10.1007/s00213-002-1030-5

Castellucci, G. A., Calbick, D., & McCormick, D. (2019). The temporal organization of mouse ultrasonic vocalizations. *PLoS ONE*, *13*(10). https://doi.org/10.1371/journal.pone.0199929

Center for Disease Control. (2016). *Module 5: Assessing and Addressing Opioid Use Disorder (OUD)*.

Ceruso, A., Martínez-Cengotitabengoa, M., Peters-Corbett, A., Diaz-Gutierrez, M. J., & Martínez-Cengotitabengoa, M. (2020). Alterations of the HPA Axis Observed in Patients with Major Depressive Disorder and Their Relation to Early Life Stress: A Systematic Review. In *Neuropsychobiology* (Vol. 79, Issue 6, pp. 417–427). https://doi.org/10.1159/000506484

Chaarani, B., Hahn, S., Allgaier, N., Adise, S., Owens, M. M., Juliano, A. C., Yuan, D. K., Loso, H., Ivanciu, A., Albaugh, M. D., Dumas, J., Mackey, S., Laurent, J., Ivanova, M., Hagler, D. J., Cornejo, M. D., Hatton, S., Agrawal, A., Aguinaldo, L., … Garavan, H. P. (2021). Baseline brain function in the preadolescents of the ABCD Study. *Nature Neuroscience*, *24*(8), 1176–1186. https://doi.org/10.1038/s41593-021-00867-9

Charles, M. K., Cooper, W. O., Jansson, L. M., Dudley, J., Slaughter, J. C., & Patrick, S. W. (2017). Male Sex Associated With Increased Risk of Neonatal Abstinence Syndrome. *Hospital Pediatrics*, *7*(6), 328–334. https://doi.org/10.1542/hpeds.2016-0218

Che, Y., Sun, H., Tan, H., Peng, Y., Zeng, T., & Ma, Y. (2005). The effect of prenatal morphine exposure on memory consolidation in the chick. *Neuroscience Letters*, *380*(3), 300–304. https://doi.org/10.1016/j.neulet.2005.01.061

Chemaly, N., Nehlig, A., Chiron, C., & Nabbout, R. (2018). Electrocorticographic telemetric recording in unrestrained mouse pups. *Journal of Neuroscience Methods*, *305*, 17–27. https://doi.org/10.1016/j.jneumeth.2018.04.020

Chiang, Y. C., Hung, T. W., & Ho, I. K. (2014a). Development of sensitization to methamphetamine in offspring prenatally exposed to morphine, methadone and buprenorphine. *Addiction Biology*, *19*(4), 676–686. https://doi.org/10.1111/adb.12055

Chiang, Y. C., Hung, T. W., & Ho, I. K. (2014b). Development of sensitization to methamphetamine in offspring prenatally exposed to morphine, methadone and buprenorphine. *Addiction Biology*, *19*(4), 676–686. https://doi.org/10.1111/adb.12055

Chiang, Y. C., Hung, T. W., Lee, C. W. S., Yan, J. Y., & Ho, I. K. (2010). Enhancement of tolerance development to morphine in rats prenatally exposed to morphine, methadone, and buprenorphine. *Journal of Biomedical Science*, *17*, 46. https://doi.org/10.1186/1423-0127-17-46

Chiou, L. C., Yeh, G. C., Fan, S. H., How, C. H., Chuang, K. C., & Tao, P. L. (2003). Prenatal morphine exposure decreases analgesia but not K+ channel activation. *NeuroReport*, *14*(2), 239–242. https://doi.org/10.1097/00001756-200302100-00016

Chong, R. Y., Oswald, L., Yang, X., Uhart, M., Lin, P. I., & Wand, G. S. (2006). The mu-opioid receptor polymorphism A118G predicts cortisol responses to naloxone and stress. *Neuropsychopharmacology*, *31*(1), 204–211. https://doi.org/10.1038/sj.npp.1300856

Ciccarone, D. (2021). The rise of illicit fentanyls, stimulants and the fourth wave of the opioid overdose crisis. In *Current Opinion in Psychiatry* (Vol. 34, Issue 4, pp. 344–350). Lippincott Williams and Wilkins. https://doi.org/10.1097/YCO.0000000000000717

Clancy, B., Darlington, R. B., & Finlay, B. L. (2001). Translating developmental time across mammalian species. *Neuroscience*, *105*(1), 7–17. https://doi.org/10.1016/S0306-4522(01)00171-3

Clarkson, J., & Herbison, A. E. (2016). Hypothalamic control of the male neonatal testosterone surge. In *Philosophical Transactions of the Royal Society B: Biological Sciences* (Vol. 371, Issue 1688). Royal Society of London. https://doi.org/10.1098/rstb.2015.0115

Clemans-Cope, L., Lynch, V., Howell, E., Hill, I., Holla, N., Morgan, J., Johnson, P., Cross-Barnet, C., & Thompson, J. A. (2019). Pregnant women with opioid use disorder and their infants in three state Medicaid programs in 2013–2016. *Drug and Alcohol Dependence*, *195*, 156–163. https://doi.org/10.1016/j.drugalcdep.2018.12.005

Cohen-Addad, N., Chatterjee, M., Bekersky, I., & Blumenthal, H. P. (1986). In utero-exposure to saccharin: A threat? *Cancer Letters*, *32*(2), 151–154. https://doi.org/10.1016/0304-3835(86)90113-8

Collins, L. R., Hall, R. W., Dajani, N. K., Wendel, P. J., Lowery, C. L., & Kay, H. H. (2005). Prolonged morphine exposure in utero causes fetal and placental vasoconstriction: A case report. *Journal of Maternal-Fetal and Neonatal Medicine*, *17*(6), 417–421. https://doi.org/10.1080/14767050500123996

Columba-Cabezas, S., Iaffaldano, G., Chiarotti, F., Alleva, E., & Cirulli, F. (2009). Early handling increases susceptibility to experimental autoimmune encephalomyelitis (EAE) in C57BL/6 male mice. *Journal of Neuroimmunology*, *212*(1–2), 10–16. https://doi.org/10.1016/j.jneuroim.2009.05.007

Comer, S., Cunningham, C., Fishman, M. J., Gordon, A., Kampman, K., Langleben, D., Nordstrom, B., Oslin, D., Woody, G., Wright, T., Wyatt, S., Femino, J., Jarvis, M., Kotz, M., Pirard, S., Roose, R. J., Geier-Horan, A., Staff, A., Haynes, B., … Woodworth, A. (n.d.). *National Practice Guideline for the Use of Medications in the Treatment of Addiction Involving Opioid Use ASAM National Practice Guideline for the Use of Medications in the Treatment of Addiction Involving Opioid Use Guideline Committee Members (alpha or*.

Conrad, C. D. (2010). A critical review of chronic stress effects on spatial learning and memory. In *Progress in Neuro-Psychopharmacology and Biological Psychiatry* (Vol. 34, Issue 5, pp. 742–755). Elsevier. https://doi.org/10.1016/j.pnpbp.2009.11.003

Conradt, E., Flannery, T., Aschner, J. L., Annett, R. D., Croen, L. A., Duarte, C. S., Friedman, A. M., Guille, C., Hedderson, M. M., Hofheimer, J. A., Jones, M. R., Ladd-Acosta, C., McGrath, M., Moreland, A., Neiderhiser, J. M., Nguyen, R. H. N., Posner, J., Ross, J. L., Savitz, D. A., … Lester, B. M. (2019). Prenatal opioid exposure: Neurodevelopmental consequences and future research priorities. *Pediatrics*, *144*(3). https://doi.org/10.1542/peds.2019-0128

Contarino, A., Picetti, R., Matthes, H. W., Koob, G. F., Kieffer, B. L., & Gold, L. H. (2002). Lack of reward and locomotor stimulation induced by heroin in μ-opioid receptor-deficient mice. *European Journal of Pharmacology*, *446*(1–3), 103–109. https://doi.org/10.1016/S0014-2999(02)01812-5

Contet, C., Kim, A., Le, D., Iyengar, S. K., Kotzebue, R. W., Yuan, C. J., Kieffer, B. L., & Mandyam, C. D. (2014). μ-Opioid receptors mediate the effects of chronic ethanol binge drinking on the hippocampal neurogenic niche. *Addiction Biology*, *19*(5), 770–780. https://doi.org/10.1111/adb.12040

Corbett, A. D., Henderson, G., McKnight, A. T., & Paterson, S. J. (2006). 75 Years of opioid research: The exciting but vain quest for the Holy Grail. In *British Journal of Pharmacology* (Vol. 147, Issue SUPPL. 1). https://doi.org/10.1038/sj.bjp.0706435

Corder, G., Castro, D. C., Bruchas, M. R., & Scherrer, G. (2018). Endogenous and exogenous opioids in pain. In *Annual Review of Neuroscience* (Vol. 41, pp. 453–473). Annual Reviews Inc. https://doi.org/10.1146/annurev-neuro-080317-061522

Corr, T. E., Schaefer, E. W., & Paul, I. M. (2018). Growth during the first year in infants affected by neonatal abstinence syndrome. *BMC Pediatrics*, *18*(1). https://doi.org/10.1186/s12887-018-1327-0

Corsi, D. J., Hsu, H., Fell, D. B., Wen, S. W., & Walker, M. (2020). Association of Maternal Opioid Use in Pregnancy with Adverse Perinatal Outcomes in Ontario, Canada, from 2012 to 2018. *JAMA Network Open*, *3*(7). https://doi.org/10.1001/jamanetworkopen.2020.8256

Craig, A., Luo, N. L., Beardsley, D. J., Wingate-Pearse, N., Walker, D. W., Hohimer, A. R., & Back, S. A. (2003a). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Experimental Neurology*, *181*(2), 231–240. https://doi.org/10.1016/S0014-4886(03)00032-3

Craig, A., Luo, N. L., Beardsley, D. J., Wingate-Pearse, N., Walker, D. W., Hohimer, A. R., & Back, S. A. (2003b). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Experimental Neurology*, *181*(2), 231–240. https://doi.org/10.1016/S0014-4886(03)00032-3

Crawley, J. N. (1999). Behavioral phenotyping of transgenic and knockout mice: Experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Research*, *835*(1), 18–26. https://doi.org/10.1016/S0006-8993(98)01258-X

Dacher, M., & Nugent, F. S. (2011). Opiates and plasticity. In *Neuropharmacology* (Vol. 61, Issue 7, pp. 1088–1096). Pergamon. https://doi.org/10.1016/j.neuropharm.2011.01.028

D'Amato, F. R. (2021). Evaluation of μ-opioid system functionality in mouse pups: Ultrasonic vocalizations as an index of infant attachment. In *Methods in Molecular Biology* (Vol. 2201, pp. 259–265). Humana Press Inc. https://doi.org/10.1007/978-1-0716-0884-5_24

Darcq, E., & Kieffer, B. L. (2018a). Opioid receptors: Drivers to addiction? In *Nature Reviews Neuroscience* (Vol. 19, Issue 8, pp. 499–514). Nature Publishing Group. https://doi.org/10.1038/s41583-018-0028-x

Darcq, E., & Kieffer, B. L. (2018b). Opioid receptors: Drivers to addiction? In *Nature Reviews Neuroscience* (Vol. 19, Issue 8, pp. 499–514). Nature Publishing Group. https://doi.org/10.1038/s41583-018-0028-x

Darevsky, D., Gill, T. M., Vitale, K. R., Hu, B., Wegner, S. A., & Hopf, F. W. (2019). Drinking despite adversity: behavioral evidence for a head down and push strategy of

conflict-resistant alcohol drinking in rats. *Addiction Biology*, *24*(3), 426–437. https://doi.org/10.1111/adb.12608

Darnaudéry, M., Louvart, H., Defrance, L., Léonhardt, M., Morley-Fletcher, S., Gruber, S. H., Galietta, G., Mathé, A. A., & Maccari, S. (2007). Impact of an intense stress on ethanol consumption in female rats characterized by their pre-stress preference: Modulation by prenatal stress. *Brain Research*, *1131*(1), 181–186. https://doi.org/10.1016/j.brainres.2006.11.005

Davidson, D., Swift, R., & Fitz, E. (1996). Naltrexone increases the latency to drink alcohol in social drinkers. *Alcoholism: Clinical and Experimental Research*, *20*(4), 732–739. https://doi.org/10.1111/j.1530-0277.1996.tb01679.x

Davis, D. D., & Templer, D. I. (1988). Neurobehavioral functioning in children exposed to narcotics in Utero. *Addictive Behaviors*, *13*(3), 275–283. https://doi.org/10.1016/0306-4603(88)90054-8

Davis, J., Eyre, H., Jacka, F. N., Dodd, S., Dean, O., McEwen, S., Debnath, M., McGrath, J., Maes, M., Amminger, P., McGorry, P. D., Pantelis, C., & Berk, M. (2016). A review of vulnerability and risks for schizophrenia: Beyond the two hit hypothesis. In *Neuroscience and Biobehavioral Reviews* (Vol. 65, pp. 185–194). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2016.03.017

Davis, J. M., Shenberger, J., Terrin, N., Breeze, J. L., Hudak, M., Wachman, E. M., Marro, P., Oliveira, E. L., Harvey-Wilkes, K., Czynski, A., Engelhardt, B., D'Apolito, K., Bogen, D., & Lester, B. (2018). Comparison of safety and efficacy of methadone vs morphine for treatment of neonatal abstinence syndrome a randomized clinical trial. *JAMA Pediatrics*, *172*(8), 741–748. https://doi.org/10.1001/jamapediatrics.2018.1307

Dayer, L. E., Painter, J. T., McCain, K., King, J., Cullen, J., & Foster, H. R. (2019). A recent history of opioid use in the US: Three decades of change. In *Substance Use and Misuse* (Vol. 54, Issue 2, pp. 331–339). Taylor and Francis Ltd. https://doi.org/10.1080/10826084.2018.1517175

De La Peña, J. B., Ahsan, H. M., Tampus, R., Botanas, C. J., Dela Peña, I. J., Kim, H. J., Sohn, A., Dela Peña, I., Shin, C. Y., Ryu, J. H., & Cheong, J. H. (2015). Cigarette smoke exposure during adolescence enhances sensitivity to the rewarding effects of nicotine in adulthood, even after a long period of abstinence. *Neuropharmacology*, *99*, 9–14. https://doi.org/10.1016/j.neuropharm.2015.06.014

De Vries, T. J., Van Vliet, B. J., Hogenboom, F., Wardeh, G., Van der Laan, J. W., Mulder, A. H., & Schoffelmeer, A. N. M. (1991a). Effect of chronic prenatal morphine treatment on μ-opioid receptor-regulated adenylate cyclase activity and neurotransmitter release in rat brain slices. *European Journal of Pharmacology: Molecular Pharmacology*, *208*(2), 97–104. https://doi.org/10.1016/0922-4106(91)90059-Q

De Vries, T. J., Van Vliet, B. J., Hogenboom, F., Wardeh, G., Van der Laan, J. W., Mulder, A. H., & Schoffelmeer, A. N. M. (1991b). Effect of chronic prenatal morphine treatment on μ-opioid receptor-regulated adenylate cyclase activity and neurotransmitter

release in rat brain slices. *European Journal of Pharmacology: Molecular Pharmacology*, *208*(2), 97–104. https://doi.org/10.1016/0922-4106(91)90059-Q

Deitmer, J. W., Theparambil, S. M., Ruminot, I., Noor, S. I., & Becker, H. M. (2019). Energy Dynamics in the Brain: Contributions of Astrocytes to Metabolism and pH Homeostasis. In *Frontiers in Neuroscience* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fnins.2019.01301

Dent, C. L., & Isles, A. R. (2014). An overview of measuring impulsive behavior in mice. In *Current protocols in mouse biology* (Vol. 4, Issue 2, pp. 35–45). Curr Protoc Mouse Biol. https://doi.org/10.1002/9780470942390.mo140015

Dess, N. K., Badia-Elder, N. E., Thiele, T. E., Kiefer, S. W., & Blizard, D. A. (1998). Ethanol consumption in rats selectively bred for differential saccharin intake. *Alcohol*, *16*(4), 275–278. https://doi.org/10.1016/S0741-8329(98)00010-X

Devlin, L. A., & Davis, J. M. (2018). A Practical Approach to Neonatal Opiate Withdrawal Syndrome. In *American Journal of Perinatology* (Vol. 35, Issue 4, pp. 324–330). Thieme Medical Publishers, Inc. https://doi.org/10.1055/s-0037-1608630

D'Hooge, R., & De Deyn, P. P. (2001). Applications of the Morris water maze in the study of learning and memory. In *Brain Research Reviews* (Vol. 36, Issue 1, pp. 60–90). https://doi.org/10.1016/S0165-0173(01)00067-4

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Dodge, N. C., Jacobson, J. L., & Jacobson, S. W. (2019). Effects of Fetal Substance Exposure on Offspring Substance Use. In *Pediatric Clinics of North America* (Vol. 66, Issue 6, pp. 1149–1161). W.B. Saunders. https://doi.org/10.1016/j.pcl.2019.08.010

Domjan, M., & Gillan, D. (1976). Role of novelty in the aversion for increasingly concentrated saccharin solutions. *Physiology and Behavior*, *16*(5), 537–542. https://doi.org/10.1016/0031-9384(76)90211-0

Drews, E., & Zimmer, A. (2010). Modulation of alcohol and nicotine responses through the endogenous opioid system. In *Progress in Neurobiology* (Vol. 90, Issue 1, pp. 1–15). Pergamon. https://doi.org/10.1016/j.pneurobio.2009.09.004

Dulor Finkler, A., Espinoza Pardo, G. V., & Bolten Lucion, A. (2020). Repeated cross-fostering affects maternal behavior and olfactory preferences in rat pups. *Developmental Psychobiology*, *62*(3), 283–296. https://doi.org/10.1002/dev.21907

Dutriez-Casteloot, I., Bernet, F., Dedieu, J. F., Croix, D., Laborie, C., Montel, V., Lesage, J., Beauvillain, J. C., & Dupouy, J. P. (1999). Hypothalamic-pituitary-adrenocortical and gonadal axes and sympathoadrenal activity of adult male rats prenatally exposed to morphine. *Neuroscience Letters*, *263*(1), 1–4. https://doi.org/10.1016/S0304-3940(99)00086-5

Eastwood, E. C., Barkley-Levenson, A. M., & Phillips, T. J. (2014). Methamphetamine drinking microstructure in mice bred to drink high or low amounts of methamphetamine. *Behavioural Brain Research*, *272*, 111–120. https://doi.org/10.1016/j.bbr.2014.06.035

Eastwood, E. C., & Phillips, T. J. (2014). Morphine intake and the effects of naltrexone and buprenorphine on the acquisition of methamphetamine intake. *Genes, Brain and Behavior*, *13*(2), 226–235. https://doi.org/10.1111/gbb.12100

Eichenbaum, G., Göhler, K., Etropolski, M., Steigerwald, I., Pergolizzi, J., Kim, M., & Vorsanger, G. (2015). Does tapentadol affect sex hormone concentrations differently from morphine and oxycodone? An initial assessment and possible implications for opioid-induced androgen deficiency. *Journal of Opioid Management*, *11*(3), 211–227. https://doi.org/10.5055/jom.2015.0270

Eichenbaum, H. (2017). Prefrontal-hippocampal interactions in episodic memory. In *Nature Reviews Neuroscience* (Vol. 18, Issue 9, pp. 547–558). Nature Publishing Group. https://doi.org/10.1038/nrn.2017.74

Eidson, L. N., & Murphy, A. Z. (2019). Inflammatory mediators of opioid tolerance: Implications for dependency and addiction. In *Peptides* (Vol. 115, pp. 51–58). Elsevier Inc. https://doi.org/10.1016/j.peptides.2019.01.003

El-Kordi, A., Winkler, D., Hammerschmidt, K., Kästner, A., Krueger, D., Ronnenberg, A., Ritter, C., Jatho, J., Radyushkin, K., Bourgeron, T., Fischer, J., Brose, N., & Ehrenreich, H. (2013). Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. *Behavioural Brain Research*, *251*, 41–49. https://doi.org/10.1016/j.bbr.2012.11.016

Eriksson, P. S., & Rönnbäck, L. (1989). Effects of prenatal morphine treatment of rats on mortality, bodyweight and analgesic response in the offspring. *Drug and Alcohol Dependence*, *24*(3), 187–194. https://doi.org/10.1016/0376-8716(89)90055-0

Ersche, K. D., Meng, C., Ziauddeen, H., Stochl, J., Williams, G. B., Bullmore, E. T., & Robbins, T. W. (2020). Brain networks underlying vulnerability and resilience to drug addiction. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(26), 15253–15261. https://doi.org/10.1073/pnas.2002509117

Esposito, G., Nakazawa, J., Venuti, P., & Bornstein, M. H. (2013). Componential deconstruction of infant distress vocalizations via tree-based models: A study of cry in autism spectrum disorder and typical development. *Research in Developmental Disabilities*, *34*(9), 2717–2724. https://doi.org/10.1016/j.ridd.2013.05.036

Falcon, E., Browne, C. A., Leon, R. M., Fleites, V. C., Sweeney, R., Kirby, L. G., & Lucki, I. (2016). Antidepressant-like effects of buprenorphine are mediated by kappa opioid receptors. *Neuropsychopharmacology*, *41*(9). https://doi.org/10.1038/npp.2016.38

Fanselow, M. S., & Cramer, C. P. (1988). The ontogeny of opiate tolerance and withdrawal in infant rats. *Pharmacology, Biochemistry and Behavior*, *31*(2), 431–438. https://doi.org/10.1016/0091-3057(88)90370-X

FDA. (2019). *Enrichment Strategies for Clinical Trials to Support Determination of Effectiveness of Human Drugs and Biological Products. Guidance for Industry*. *March*, 1–41.

Feghali, M., Venkataramanan, R., & Caritis, S. (2015). Pharmacokinetics of drugs in pregnancy. In *Seminars in Perinatology* (Vol. 39, Issue 7, pp. 512–519). W.B. Saunders. https://doi.org/10.1053/j.semperi.2015.08.003

Ferraro, T. N., Golden, G. T., Smith, G. G., Martin, J. F., Schwebel, C. L., Doyle, G. A., Buono, R. J., & Berrettini, W. H. (2005a). Confirmation of a major QTL influencing oral morphine intake in C57 and DBA mice using reciprocal congenic strains. *Neuropsychopharmacology*, *30*(4), 742–746. https://doi.org/10.1038/sj.npp.1300592

Ferraro, T. N., Golden, G. T., Smith, G. G., Martin, J. F., Schwebel, C. L., Doyle, G. A., Buono, R. J., & Berrettini, W. H. (2005b). Confirmation of a major QTL influencing oral morphine intake in C57 and DBA mice using reciprocal congenic strains. *Neuropsychopharmacology*, *30*(4), 742–746. https://doi.org/10.1038/sj.npp.1300592

Fields, H. L., & Margolis, E. B. (2015). Understanding opioid reward. In *Trends in Neurosciences* (Vol. 38, Issue 4, pp. 217–225). Elsevier Ltd. https://doi.org/10.1016/j.tins.2015.01.002

Fleites, V. C., Markwalter, P. S., Johnson, K., & De Biasi, M. (2022). In utero exposure to morphine leads to sex-specific behavioral alterations that persist into adulthood in cross-fostered mice. *BioRxiv*, 2022.02.28.482336. https://doi.org/10.1101/2022.02.28.482336

Fleming, D. A. (2018). Prescription Opioid Misuse and Overuse in the U.S. *Missouri Medicine*, *115*(3), 189–190.

Ford, C. P., Mark, G. P., & Williams, J. T. (2006). Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. *Journal of Neuroscience*, *26*(10), 2788–2797. https://doi.org/10.1523/JNEUROSCI.4331-05.2006

Fox, W. M. (1965). Reflex-ontogeny and behavioural development of the mouse. *Animal Behaviour*, *13*(2–3). https://doi.org/10.1016/0003-3472(65)90041-2

Frances, B., Gout, R., Monsarrat, B., Cros, J., & Zajac, J. M. (1992). Further evidence that morphine-6β-glucuronide is a more potent opioid agonist than morphine. *Journal of Pharmacology and Experimental Therapeutics*, *262*(1), 25–31. https://doi.org/10.1097/00132586-199304000-00005

Friedman, J., & Akre, S. (2021). COVID-19 and the Drug Overdose Crisis: Uncovering the Deadliest Months in the United States, January–July 2020. *American Journal of Public Health*, *111*(7), 1284–1291. https://doi.org/10.2105/AJPH.2021.306256

Fujita-Hamabe, W., Nishida, M., Nawa, A., Kobori, T., Nakamoto, K., Kishioka, S., & Tokuyama, S. (2012). Etoposide modulates the effects of oral morphine analgesia by targeting the intestinal P-glycoprotein. *Journal of Pharmacy and Pharmacology*, *64*(4), 496–504. https://doi.org/10.1111/j.2042-7158.2011.01426.x

Gagin, R., Kook, N., Cohen, E., & Shavit, Y. (1997). Prenatal morphine enhances morphine-conditioned place preference in adult rats. *Pharmacology Biochemistry and Behavior*, *58*(2), 525–528. https://doi.org/10.1016/S0091-3057(97)00281-5

Galeote, L., Berrendero, F., Andreea Bura, S., Zimmer, A., & Maldonado, R. (2009). Prodynorphin gene disruption increases the sensitivity to nicotine self-administration in mice. *International Journal of Neuropsychopharmacology*, *12*(5), 615–625. https://doi.org/10.1017/S1461145708009450

Galeote, L., Kieffer, B. L., Maldonado, R., & Berrendero, F. (2006). Mu-opioid receptors are involved in the tolerance to nicotine antinociception. *Journal of Neurochemistry*, *97*(2), 416–423. https://doi.org/10.1111/j.1471-4159.2006.03751.x

Gan, W. Q., Cohen, S. B. Z., Paul Man, S. F., & Sin, D. D. (2008). Sex-related differences in serum cotinine concentrations in daily cigarette smokers. *Nicotine and Tobacco Research*, *10*(8), 1293–1300. https://doi.org/10.1080/14622200802239132

Gangitano, D., Salas, R., Teng, Y., Perez, E., & De Biasi, M. (2009). Progesterone modulation of α5 nAChR subunits influences anxiety-related behavior during estrus cycle. *Genes, Brain and Behavior*, *8*(4), 398–406. https://doi.org/10.1111/j.1601-183X.2009.00476.x

Garland, M., Abildskov, K. M., Kiu, T. W., Daniel, S. S., Weldy, P., & Stark, R. I. (2008). Placental transfer and fetal elimination of morphine-3-β-glucuronide in the pregnant baboon. *Drug Metabolism and Disposition*, *36*(9), 1859–1868. https://doi.org/10.1124/dmd.108.021352

Gauthier, A. C., Deangeli, N. E., & Bucci, D. J. (2015). Cross-fostering differentially affects ADHD-related behaviors in spontaneously hypertensive rats. *Developmental Psychobiology*, *57*(2), 226–236. https://doi.org/10.1002/dev.21286

Gavériaux-Ruff, C., & Kieffer, B. L. (2002). Opioid receptor genes inactivated in mice: The highlights. *Neuropeptides*, *36*(2–3), 62–71. https://doi.org/10.1054/npep.2002.0900

Gerdin, E., Rane, A., & Lindberg, B. (1990). Transplacental transfer of morphine in man. *Journal of Perinatal Medicine*, *18*(4), 305–312. https://doi.org/10.1515/jpme.1990.18.4.305

Ghose, R., Forati, A. M., & Mantsch, J. R. (2022). Impact of the COVID-19 Pandemic on Opioid Overdose Deaths: a Spatiotemporal Analysis. *Journal of Urban Health*, *99*(2), 316–327. https://doi.org/10.1007/s11524-022-00610-0

Gianoulakis, C. (2001). Influence of the endogenous opioid system on high alcohol consumption and genetic predisposition to alcoholism. In *Journal of Psychiatry and Neuroscience* (Vol. 26, Issue 4, pp. 304–318). Canadian Medical Association.

Gianoulakis, C., Béliveau, D., Angelogianni, P., Meaney, M., Thavundayil, J., Tawar, V., & Dumas, M. (1989). Different pituitary ß-endorphin and adrenal cortisol response to ethanol in individuals with high and low risk for future development of alcoholism. *Life Sciences*, *45*(12), 1097–1109. https://doi.org/10.1016/0024-3205(89)90167-7

Girirajan, S., & Eichler, E. E. (2010). Phenotypic variability and genetic susceptibility to genomic disorders. *Human Molecular Genetics*, *19*(R2), R176. https://doi.org/10.1093/hmg/ddq366

Glantz, M. D., & Chambers, J. C. (2006). Prenatal drug exposure effects on subsequent vulnerability to drug abuse. In *Development and Psychopathology* (Vol. 18, Issue 3, pp. 893–922). Cambridge University Press. https://doi.org/10.1017/S0954579406060445

Glendinning, J. I. (2018). Oral Post-Oral Actions of Low-Calorie Sweeteners: A Tale of Contradictions and Controversies. *Obesity*, *26*(S3), S9–S17. https://doi.org/10.1002/oby.22253

Glick, S. D., Strumpf, A. J., & Zimmerberg, B. (1977a). Effect of in utero administration of morphine on the subsequent development of self-administration behavior. *Brain Research*, *132*(1), 194–196. https://doi.org/10.1016/0006-8993(77)90720-X

Glick, S. D., Strumpf, A. J., & Zimmerberg, B. (1977b). Effect of in utero administration of morphine on the subsequent development of self-administration behavior. *Brain Research*, *132*(1), 194–196. https://doi.org/10.1016/0006-8993(77)90720-X

Godynyuk, E., Bluitt, M. N., Tooley, J. R., Kravitz, A. V., & Creed, M. C. (2019). An open-source, automated home-cage sipper device for monitoring liquid ingestive behavior in rodents. *ENeuro*, *6*(5). https://doi.org/10.1523/ENEURO.0292-19.2019

Goetzl, L., Thompson-Felix, T., Darbinian, N., Merabova, N., Merali, S., Merali, C., Sanserino, K., Tatevosian, T., Fant, B., & Wimmer, M. E. (2019). Novel biomarkers to assess in utero effects of maternal opioid use: First steps toward understanding short- and long-term neurodevelopmental sequelae. *Genes, Brain and Behavior*, *18*(6), e12583. https://doi.org/10.1111/gbb.12583

Golden, S. A., Jin, M., & Shaham, Y. (2019). Animal models of (or for) aggression reward, addiction, and relapse: Behavior and circuits. *Journal of Neuroscience*, *39*(21), 3996–4008. https://doi.org/10.1523/JNEUROSCI.0151-19.2019

Goldstein, D. S. (2010). Adrenal responses to stress. *Cellular and Molecular Neurobiology*, *30*(8), 1433–1440. https://doi.org/10.1007/s10571-010-9606-9

Gong, W., Rolls, E. T., Du, J., Feng, J., & Cheng, W. (2021). Brain structure is linked to the association between family environment and behavioral problems in children in the ABCD study. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-021-23994-0

Goodwin, G. A., & Barr, G. A. (2005). Developmental changes in the behavioral and autonomic effects of kappa opioid receptor stimulation of the midbrain periaqueductal gray. *Developmental Psychobiology*, *46*(1), 47–56. https://doi.org/10.1002/dev.20039

Goran, M. I., Plows, J. F., & Ventura, E. E. (2019). Effects of consuming sugars and alternative sweeteners during pregnancy on maternal and child health: Evidence for a secondhand sugar effect. *Proceedings of the Nutrition Society*, *78*(3), 262–271. https://doi.org/10.1017/S002966511800263X

Grecco, G. G., Haggerty, D. L., Reeves, K. C., Gao, Y., Maulucci, D., & Atwood, B. K. (2022). Prenatal opioid exposure reprograms the behavioural response to future alcohol reward. *Addiction Biology*, *27*(2), e13136. https://doi.org/10.1111/adb.13136

Griffiths, S. K., & Campbell, J. P. (2015). Placental structure, function and drug transfer. *Continuing Education in Anaesthesia, Critical Care and Pain*, *15*(2), 84–89. https://doi.org/10.1093/bjaceaccp/mku013

Grigson, P. S., Twining, R. C., & Carelli, R. M. (2000). Heroin-induced suppression of saccharin intake in water-deprived and water-replete rats. *Pharmacology Biochemistry and Behavior*, *66*(3), 603–608. https://doi.org/10.1016/S0091-3057(00)00253-7

Grimaud, J., & Murthy, V. N. (2018). How to monitor breathing in laboratory rodents: A review of the current methods. In *Journal of Neurophysiology* (Vol. 120, Issue 2, pp. 624–632). American Physiological Society. https://doi.org/10.1152/jn.00708.2017

Gui, L., Loukas, S., Lazeyras, F., Hüppi, P. S., Meskaldji, D. E., & Borradori Tolsa, C. (2019). Longitudinal study of neonatal brain tissue volumes in preterm infants and their ability to predict neurodevelopmental outcome. *NeuroImage*, *185*, 728–741. https://doi.org/10.1016/j.neuroimage.2018.06.034

Gulemetova, R., & Kinkead, R. (2011). Neonatal stress increases respiratory instability in rat pups. *Respiratory Physiology and Neurobiology*, *176*(3), 103–109. https://doi.org/10.1016/j.resp.2011.01.014

Gur, R. E., Moore, T. M., Rosen, A. F. G., Barzilay, R., Roalf, D. R., Calkins, M. E., Ruparel, K., Scott, J. C., Almasy, L., Satterthwaite, T. D., Shinohara, R. T., & Gur, R. C. (2019). Burden of Environmental Adversity Associated with Psychopathology, Maturation, and Brain Behavior Parameters in Youths. *JAMA Psychiatry*, *76*(9), 966–975. https://doi.org/10.1001/jamapsychiatry.2019.0943

Gururajan, A., van de Wouw, M., Boehme, M., Becker, T., O'Connor, R., Bastiaanssen, T. F. S., Moloney, G. M., Lyte, J. M., Ventura Silva, A. P., Merckx, B., Dinan, T. G., & Cryan, J. F. (2019). Resilience to chronic stress is associated with specific neurobiological, neuroendocrine and immune responses. *Brain, Behavior, and Immunity*, *80*, 583–594. https://doi.org/10.1016/j.bbi.2019.05.004

Guyenet, S. J., Furrer, S. A., Damian, V. M., Baughan, T. D., la Spada, A. R., & Garden, G. A. (2010). A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia. *Journal of Visualized Experiments*, *39*. https://doi.org/10.3791/1787

Haapasalo, J., Nordfors, K., Haapasalo, H., & Parkkila, S. (2020). The expression of carbonic anhydrases II, IX and XII in brain tumors. In *Cancers* (Vol. 12, Issue 7, pp. 1–20). MDPI AG. https://doi.org/10.3390/cancers12071723

Hagler, D. J., Hatton, S. N., Cornejo, M. D., Makowski, C., Fair, D. A., Dick, A. S., Sutherland, M. T., Casey, B. J., Barch, D. M., Harms, M. P., Watts, R., Bjork, J. M., Garavan, H. P., Hilmer, L., Pung, C. J., Sicat, C. S., Kuperman, J., Bartsch, H., Xue, F., … Dale, A. M. (2019). Image processing and analysis methods for the Adolescent Brain

Cognitive Development Study. *NeuroImage*, *202*. https://doi.org/10.1016/j.neuroimage.2019.116091

Hahn, M. E., & Lavooy, M. J. (2005). A review of the methods of studies on infant ultrasound production and maternal retrieval in small rodents. *Behavior Genetics*, *35*(1), 31–52. https://doi.org/10.1007/s10519-004-0854-7

Hall, F. S., Sora, I., & Uhl, G. R. (2001). Ethanol consumption and reward are decreased in μ-opiate receptor knockout mice. *Psychopharmacology*, *154*(1), 43–49. https://doi.org/10.1007/s002130000622

Hall, W., & Weier, M. (2017). Lee Robins' studies of heroin use among US Vietnam veterans. *Addiction*, *112*(1), 176–180. https://doi.org/10.1111/add.13584

Hammond, D., Reid, J. L., Burkhalter, R., O'Connor, R. J., Goniewicz, M. L., Wackowski, O. A., Thrasher, J. F., & Hitchman, S. C. (2021). Trends in e-cigarette brands, devices and the nicotine profile of products used by youth in England, Canada and the USA: 2017-2019. *Tobacco Control*. https://doi.org/10.1136/tobaccocontrol-2020-056371

Hannibal, K. E., & Bishop, M. D. (2014). Chronic stress, cortisol dysfunction, and pain: A psychoneuroendocrine rationale for stress management in pain rehabilitation. *Physical Therapy*, *94*(12), 1816–1825. https://doi.org/10.2522/ptj.20130597

Harburg, G. C., Hall, F. S., Harrist, A. V., Sora, I., Uhl, G. R., & Eisch, A. J. (2007). Knockout of the mu opioid receptor enhances the survival of adult-generated hippocampal granule cell neurons. *Neuroscience*, *144*(1), 77–87. https://doi.org/10.1016/j.neuroscience.2006.09.018

Hauser, K. F., & Knapp, P. E. (2018). Opiate drugs with abuse liability hijack the endogenous opioid system to disrupt neuronal and glial maturation in the central nervous system. In *Frontiers in Pediatrics* (Vol. 5, p. 1). Frontiers Media S.A. https://doi.org/10.3389/fped.2017.00294

Havens, J. R., Simmons, L. A., Shannon, L. M., & Hansen, W. F. (2009). Factors associated with substance use during pregnancy: Results from a national sample. *Drug and Alcohol Dependence*, *99*(1–3), 89–95. https://doi.org/10.1016/j.drugalcdep.2008.07.010

He, X., Bao, Y., Li, Y., & Sui, N. (2010). The effects of morphine at different embryonic ages on memory consolidation and rewarding properties of morphine in day-old chicks. *Neuroscience Letters*, *482*(1), 12–16. https://doi.org/10.1016/j.neulet.2010.06.074

Hedegaard, H., Curtin, S. C., & Warner, M. (2018). Suicide Mortality in the United States, 1999-2017. *NCHS Data Brief*, *330*, 1–8.

Hemmati, Z., Conti, A. A., & Baldacchino, A. (2022). Ophthalmic outcomes in children exposed to opioid maintenance treatment in utero: A systematic review and meta-analysis. In *Neuroscience and Biobehavioral Reviews* (Vol. 136, p. 104601). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2022.104601

Hermann, D., Hirth, N., Reimold, M., Batra, A., Smolka, M. N., Hoffmann, S., Kiefer, F., Noori, H. R., Sommer, W. H., Reischl, G., La Fougère, C., Mann, K., Spanagel, R., & Hansson, A. C. (2017). Low μ-Opioid Receptor Status in Alcohol Dependence Identified by Combined Positron Emission Tomography and Post-Mortem Brain Analysis. *Neuropsychopharmacology*, *42*(3), 606–614. https://doi.org/10.1038/npp.2016.145

Higgins, S. T., Kurti, A. N., Palmer, M., Tidey, J. W., Cepeda-Benito, A., Cooper, M. R., Krebs, N. M., Baezconde-Garbanati, L., Hart, J. L., & Stanton, C. A. (2019). A review of tobacco regulatory science research on vulnerable populations. *Preventive Medicine*, *128*. https://doi.org/10.1016/j.ypmed.2019.04.024

Hirai, A. H., Ko, J. Y., Owens, P. L., Stocks, C., & Patrick, S. W. (2021). Neonatal Abstinence Syndrome and Maternal Opioid-Related Diagnoses in the US, 2010-2017. *JAMA - Journal of the American Medical Association*, *325*(2), 146–155. https://doi.org/10.1001/jama.2020.24991

Holsapple, M. P., West, L. J., & Landreth, K. S. (2003). Species comparison of anatomical and functional immune system development. In *Birth Defects Research Part B - Developmental and Reproductive Toxicology* (Vol. 68, Issue 4, pp. 321–334). John Wiley & Sons, Ltd. https://doi.org/10.1002/bdrb.10035

Honeywell, K. M., Van Doren, E., & Szumlinski, K. K. (2022). Selective Inhibition of PDE4B Reduces Methamphetamine Reinforcement in Two C57BL/6 Substrains. *International Journal of Molecular Sciences*, *23*(9). https://doi.org/10.3390/ijms23094872

Houdi, A. A., Dasgupta, R., & Kindy, M. S. (1998). Effect of nicotine use and withdrawal on preproenkephalin a mRNA. *Brain Research*, *799*(2), 257–263. https://doi.org/10.1016/S0006-8993(98)00454-5

Houdi, A. A., Pierzchala, K., Marson, L., Palkovits, M., & Van Loon, G. R. (1991). Nicotine-induced alteration in Tyr-Gly-Gly and Met-enkephalin in discrete brain nuclei reflects altered enkephalin neuron activity. *Peptides*, *12*(1), 161–166. https://doi.org/10.1016/0196-9781(91)90183-P

Hwa, L. S., Chu, A., Levinson, S. A., Kayyali, T. M., Debold, J. F., & Miczek, K. A. (2011). Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. *Alcoholism: Clinical and Experimental Research*, *35*(11), 1938–1947. https://doi.org/10.1111/j.1530-0277.2011.01545.x

Hyytiä, P., Ingman, K., Soini, S. L., Laitinen, J. T., & Korpi, E. R. (1999). Effects of continuous opioid receptor blockade on alcohol intake and up- regulation of opioid receptor subtype signalling in a genetic model of high alcohol drinking. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *360*(4), 391–401. https://doi.org/10.1007/s002109900070

Interrante, J. D., Scroggs, S. L. P., Hogue, C. J., Friedman, J. M., Reefhuis, J., Jann, M. W., & Broussard, C. S. (2021). Prescription opioid use during pregnancy and risk for preterm birth or term low birthweight. In *Journal of Opioid Management* (Vol. 17, Issue 3, pp. 215–225). Weston Medical Publishing. https://doi.org/10.5055/JOM.2021.0632

Isaacs, K. R., Atreyapurapu, S., Alyusuf, A. H., Ledgerwood, D. M., Finnegan, L. P., Chang, K. H. K., Ma, T. X., & Washio, Y. (2021). Neonatal outcomes after combined opioid and nicotine exposure in utero: A scoping review. In *International Journal of Environmental Research and Public Health* (Vol. 18, Issue 19). MDPI. https://doi.org/10.3390/ijerph181910215

Jacinto, L. R., Mata, R., Novais, A., Marques, F., & Sousa, N. (2017). The habenula as a critical node in chronic stress-related anxiety. *Experimental Neurology*, *289*, 46–54. https://doi.org/10.1016/j.expneurol.2016.12.003

James, K., & Jordan, A. (2018). The opioid crisis in black communities. *Journal of Law, Medicine and Ethics*, *46*(2), 404–421. https://doi.org/10.1177/1073110518782949

Jansson, L. M., Dipietro, J. A., Elko, A., & Velez, M. (2007). Maternal vagal tone change in response to methadone is associated with neonatal abstinence syndrome severity in exposed neonates. *Journal of Maternal-Fetal and Neonatal Medicine*, *20*(9), 677–685. https://doi.org/10.1080/14767050701490327

Jantzie, L. L., Maxwell, J. R., Newville, J. C., Yellowhair, T. R., Kitase, Y., Madurai, N., Ramachandra, S., Bakhireva, L. N., Northington, F. J., Gerner, G., Tekes, A., Milio, L. A., Brigman, J. L., Robinson, S., & Allan, A. (2020). Prenatal opioid exposure: The next neonatal neuroinflammatory disease. *Brain, Behavior, and Immunity*, *84*, 45–58. https://doi.org/10.1016/j.bbi.2019.11.007

Jarjour, S., Bai, L., & Gianoulakis, C. (2009). Effect of acute ethanol administration on the release of opioid peptides from the midbrain including the ventral tegmental area. *Alcoholism: Clinical and Experimental Research*, *33*(6), 1033–1043. https://doi.org/10.1111/j.1530-0277.2009.00924.x

Jarlenski, M., Kim, J. Y., Ahrens, K. A., Allen, L., Austin, A., Barnes, A. J., Crane, D., Lanier, P., Mauk, R., Mohamoud, S., Pauly, N., Talbert, J., Zivin, K., & Donohue, J. M. (2021). Healthcare Patterns of Pregnant Women and Children Affected by OUD in 9 State Medicaid Populations. *Journal of Addiction Medicine*, *15*(5), 406–413. https://doi.org/10.1097/ADM.0000000000000780

Jarlenski, M. P., Krans, E. E., Kim, J. Y., Donohue, J. M., Everette James, A., Kelley, D., Stein, B. D., & Bogen, D. L. (2020). Five-year outcomes among medicaid-enrolled children with in utero opioid exposure. *Health Affairs*, *39*(2), 247–255. https://doi.org/10.1377/hlthaff.2019.00740

Jiang, J., He, X., Wang, M. Y., & Sui, N. (2011). Early prenatal morphine exposure impairs performance of learning tasks and attenuates in vitro heterosynaptic long-term potentiation of intermediate medial mesopallium in day-old chicks. *Behavioural Brain Research*, *219*(2), 363–366. https://doi.org/10.1016/j.bbr.2010.12.034

Jiang, Y., Peng, T., Gaur, U., Silva, M., Little, P., Chen, Z., Qiu, W., Zhang, Y., & Zheng, W. (2019). Role of corticotropin releasing factor in the neuroimmune mechanisms of depression: Examination of current pharmaceutical and herbal therapies. *Frontiers in Cellular Neuroscience*, *13*, 290. https://doi.org/10.3389/fncel.2019.00290

Job, M. O., Tang, A., Hall, F. S., Sora, I., Uhl, G. R., Bergeson, S. E., & Gonzales, R. A. (2007). Mu (μ) Opioid Receptor Regulation of Ethanol-Induced Dopamine Response in the Ventral Striatum: Evidence of Genotype Specific Sexual Dimorphic Epistasis. *Biological Psychiatry*, *62*(6), 627–634. https://doi.org/10.1016/j.biopsych.2006.11.016

Jóhannesson, T., & Becker, B. A. (1972). The Effects of Maternally–administered Morphine on Rat Foetal Development and Resultant Tolerance to the Analgesic Effect of Morphine. *Acta Pharmacologica et Toxicologica*, *31*(4), 305–313. https://doi.org/10.1111/j.1600-0773.1972.tb00686.x

Jones, H. E., Heil, S. H., Baewert, A., Arria, A. M., Kaltenbach, K., Martin, P. R., Coyle, M. G., Selby, P., Stine, S. M., & Fischer, G. (2012). Buprenorphine treatment of opioid-dependent pregnant women: A comprehensive review. In *Addiction* (Vol. 107, Issue SUPPL.1, pp. 5–27). John Wiley & Sons, Ltd. https://doi.org/10.1111/j.1360-0443.2012.04035.x

Jones, H. E., Johnson, R. E., Jasinski, D. R., O'Grady, K. E., Chisholm, C. A., Choo, R. E., Crocetti, M., Dudas, R., Harrow, C., Huestis, M. A., Jansson, L. M., Lantz, M., Lester, B. M., & Milio, L. (2005). Buprenorphine versus methadone in the treatment of pregnant opioid-dependent patients: Effects on the neonatal abstinence syndrome. *Drug and Alcohol Dependence*, *79*(1), 1–10. https://doi.org/10.1016/j.drugalcdep.2004.11.013

Jones, H. E., Kaltenbach, K., Heil, S. H., Stine, S. M., Coyle, M. G., Arria, A. M., O'Grady, K. E., Selby, P., Martin, P. R., & Fischer, G. (2010). Neonatal Abstinence Syndrome after Methadone or Buprenorphine Exposure. *New England Journal of Medicine*, *363*(24), 2320–2331. https://doi.org/10.1056/nejmoa1005359

Jones, H. E., & Kraft, W. K. (2019). Analgesia, Opioids, and Other Drug Use During Pregnancy and Neonatal Abstinence Syndrome. In *Clinics in Perinatology* (Vol. 46, Issue 2, pp. 349–366). W.B. Saunders. https://doi.org/10.1016/j.clp.2019.02.013

June, H. L., Cummings, R., Eiler, W. J. A., Foster, K. L., McKay, P. F., Seyoum, R., Garcia, M., McCane, S., Grey, C., Hawkins, S. E., & Mason, D. (2004). Central Opioid Receptors Differentially Regulate the Nalmefene-Induced Suppression of Ethanol- And Saccharin-Reinforced Behaviors in Alcohol-Preferring (P) Rats. *Neuropsychopharmacology*, *29*(2), 285–299. https://doi.org/10.1038/sj.npp.1300338

Kaltenbach, K., O'Grady, K. E., Heil, S. H., Salisbury, A. L., Coyle, M. G., Fischer, G., Martin, P. R., Stine, S., & Jones, H. E. (2018). Prenatal exposure to methadone or buprenorphine: Early childhood developmental outcomes. *Drug and Alcohol Dependence*, *185*, 40–49. https://doi.org/10.1016/j.drugalcdep.2017.11.030

Kamboj, S. K., Tookman, A., Jones, L., & Curran, V. H. (2005). The effects of immediate-release morphine on cognitive functioning in patients receiving chronic opioid therapy in palliative care. *Pain*, *117*(3), 388–395. https://doi.org/10.1016/j.pain.2005.06.022

Kamens, H. M., Silva, C., Peck, C., & Miller, C. N. (2018). Varenicline modulates ethanol and saccharin consumption in adolescent male and female C57BL/6J mice. *Brain Research Bulletin*, *138*, 20–25. https://doi.org/10.1016/j.brainresbull.2017.07.020

Kaneyasu, M. (2012). Pain management, morphine administration, and outcomes in preterm infants: A review of the literature. *Neonatal Network*, *31*(1), 21–30. https://doi.org/10.1891/0730-0832.31.1.21

Kanny, D., Naimi, T. S., Liu, Y., Lu, H., & Brewer, R. D. (2018). Annual Total Binge Drinks Consumed by U.S. Adults, 2015. *American Journal of Preventive Medicine*, *54*(4), 486–496. https://doi.org/10.1016/j.amepre.2017.12.021

Kelty, E., & Hulse, G. (2017). A retrospective cohort study of the health of children prenatally exposed to methadone, buprenorphine or naltrexone compared with non-exposed control children. *American Journal on Addictions*, *26*(8), 845–851. https://doi.org/10.1111/ajad.12642

Kibaly, C., Xu, C., Cahill, C. M., Evans, C. J., & Law, P. Y. (2019). Non-nociceptive roles of opioids in the CNS: opioids' effects on neurogenesis, learning, memory and affect. In *Nature Reviews Neuroscience* (Vol. 20, Issue 1, pp. 5–18). Nature Publishing Group. https://doi.org/10.1038/s41583-018-0092-2

Kieffer, B. L., & Gavériaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. In *Progress in Neurobiology* (Vol. 66, Issue 5, pp. 285–306). https://doi.org/10.1016/S0301-0082(02)00008-4

Kiem, D. T., Fekete, M. I., & Makara, G. B. (1995). Diurnal alteration in opiate effects on the hypothalamo-pituitary-adrenal axis: changes in the mechanism of action. *European Journal of Pharmacology*, *272*(2–3), 145–150. https://doi.org/10.1016/0014-2999(94)00639-O

Kittirattanapaiboon, P., Srikosai, S., & Wittayanookulluk, A. (2017). Methamphetamine use and dependence in vulnerable female populations. In *Current Opinion in Psychiatry* (Vol. 30, Issue 4, pp. 247–252). Lippincott Williams and Wilkins. https://doi.org/10.1097/YCO.0000000000000335

Klausz, B., Pintér, O., Sobor, M., Gyarmati, Z., Fürst, Z., Tímár, J., & Zelena, D. (2011a). Changes in adaptability following perinatal morphine exposure in juvenile and adult rats. *European Journal of Pharmacology*, *654*(2), 166–172. https://doi.org/10.1016/j.ejphar.2010.11.025

Klausz, B., Pintér, O., Sobor, M., Gyarmati, Z., Fürst, Z., Tímár, J., & Zelena, D. (2011b). Changes in adaptability following perinatal morphine exposure in juvenile and adult rats. *European Journal of Pharmacology*, *654*(2), 166–172. https://doi.org/10.1016/j.ejphar.2010.11.025

Klein, L. C., Stine, M. M., Vandenbergh, D. J., Whetzel, C. A., & Kamens, H. M. (2004). Sex differences in voluntary oral nicotine consumption by adolescent mice: A dose-response experiment. *Pharmacology Biochemistry and Behavior*, *78*(1), 13–25. https://doi.org/10.1016/j.pbb.2004.01.005

Klug, M., Hill, R., & van den Buuse, M. (2015). Two hit' neurodevelopmental mechanisms in schizophrenia: Focus on animal models and the role of BDNF. In *Psychiatry and Neuroscience Update: Bridging the Divide* (pp. 335–352). Springer International Publishing. https://doi.org/10.1007/978-3-319-17103-6_24

Koabel, J., McNivens, M., McKee, P., Pautassi, R., Bordner, K., & Nizhnikov, M. (2021). The offspring of alcohol-exposed sires exhibit heightened ethanol intake and behavioral alterations in the elevated plus maze. *Alcohol*, *92*, 65–72. https://doi.org/10.1016/j.alcohol.2021.01.009

Koebele, S. V, & Bimonte-Nelson, H. A. (2016). Modeling menopause: The utility of rodents in translational behavioral endocrinology research. In *Maturitas* (Vol. 87, pp. 5–17). https://doi.org/10.1016/j.maturitas.2016.01.015

Konkle, A. T. M., & McCarthy, M. M. (2011). Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. *Endocrinology*, *152*(1), 223–235. https://doi.org/10.1210/en.2010-0607

Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: a neurocircuitry analysis. In *The Lancet Psychiatry* (Vol. 3, Issue 8, pp. 760–773). Elsevier Ltd. https://doi.org/10.1016/S2215-0366(16)00104-8

Kosten, T. A., & Ambrosio, E. (2002). HPA axis function and drug addictive behaviors: Insights from studies with Lewis and Fischer 344 inbred rats. *Psychoneuroendocrinology*, *27*(1–2), 35–69. https://doi.org/10.1016/S0306-4530(01)00035-X

Kosten, T. R., & George, T. P. (2002). The neurobiology of opioid dependence: implications for treatment. In *Science & practice perspectives / a publication of the National Institute on Drug Abuse, National Institutes of Health* (Vol. 1, Issue 1, pp. 13–20). National Institute on Drug Abuse. https://doi.org/10.1151/spp021113

Kota, D., Robinson, S. E., & Imad Damaj, M. (2009). Enhanced nicotine reward in adulthood after exposure to nicotine during early adolescence in mice. *Biochemical Pharmacology*, *78*(7), 873–879. https://doi.org/10.1016/j.bcp.2009.06.099

Kovacs, K. M., Szakall, I., O'Brien, D., Wang, R., Vinod, K. Y., Saito, M., Simonin, F., Kieffer, B. L., & Vadasz, C. (2005). Decreased oral self-administration of alcohol in κ-opioid receptor knock-out mice. *Alcoholism: Clinical and Experimental Research*, *29*(5), 730–738. https://doi.org/10.1097/01.ALC.0000164361.62346.D6

Kranzler, H. R., Washio, Y., Zindel, L. R., Lynch, K. G., Hand, D., Tyndale, R. F., Oncken, C., & Schnoll, R. (2021). Pregnant smokers receiving opioid agonist therapy have an elevated nicotine metabolite ratio: A replication study. *Nicotine and Tobacco Research*, *22*(10), 1923–1927. https://doi.org/10.1093/NTR/NTAA066

Kreek, M. J., Reed, B., & Butelman, E. R. (2019). Current status of opioid addiction treatment and related preclinical research. In *Sci. Adv* (Vol. 5).

Krieger, H., Young, C. M., Anthenien, A. M., & Neighbors, C. (2018). The Epidemiology of Binge Drinking Among College-Age Individuals in the United States. In *Alcohol research : current reviews* (Vol. 39, Issue 1, pp. 23–30). NLM (Medline).

Krishnan, A., Samtani, R., Dhanantwari, P., Lee, E., Yamada, S., Shiota, K., Donofrio, M. T., Leatherbury, L., & Lo, C. W. (2014). A detailed comparison of mouse and human

cardiac development. *Pediatric Research*, *76*(6), 500–507. https://doi.org/10.1038/pr.2014.128

Kromkhun, P., Katou, M., Hashimoto, H., Terada, M., Moon, C., & Saito, T. R. (2013). Quantitative and qualitative analysis of rat pup ultrasonic vocalization sounds induced by a hypothermic stimulus. *Laboratory Animal Research*, *29*(2), 77. https://doi.org/10.5625/lar.2013.29.2.77

Kroon, T., van Hugte, E., van Linge, L., Mansvelder, H. D., & Meredith, R. M. (2019). Early postnatal development of pyramidal neurons across layers of the mouse medial prefrontal cortex. *Scientific Reports*, *9*(1), 1–16. https://doi.org/10.1038/s41598-019-41661-9

Kuhn, B. N., Kalivas, P. W., & Bobadilla, A. C. (2019). Understanding Addiction Using Animal Models. In *Frontiers in Behavioral Neuroscience* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fnbeh.2019.00262

Kumar, S., Hultman, R., Hughes, D., Michel, N., Katz, B. M., & Dzirasa, K. (2014). Prefrontal cortex reactivity underlies trait vulnerability to chronic social defeat stress. *Nature Communications*, *5*(1), 1–9. https://doi.org/10.1038/ncomms5537

Kvello, A. M. S., Andersen, J. M., Øiestad, E. L., Steinsland, S., Aase, A., Mørland, J., & Bogen, I. L. (2019). A monoclonal antibody against 6-acetylmorphine protects female mice offspring from adverse behavioral effects induced by prenatal heroin exposure. *Journal of Pharmacology and Experimental Therapeutics*, *368*(1), 106–115. https://doi.org/10.1124/jpet.118.251504

Kwarteng, A. E., Rahman, M. M., Gee, D. G., Infante, M. A., Tapert, S. F., & Curtis, B. L. (2021). Child reward neurocircuitry and parental substance use history: Findings from the Adolescent Brain Cognitive Development Study. *Addictive Behaviors*, *122*. https://doi.org/10.1016/j.addbeh.2021.107034

Labella, M. H., Eiden, R. D., Tabachnick, A. R., Sellers, T., & Dozier, M. (2021). Infant neurodevelopmental outcomes of prenatal opioid exposure and polysubstance use. *Neurotoxicology and Teratology*, *86*, 107000. https://doi.org/10.1016/j.ntt.2021.107000

Laborie, C., Dutriez-Casteloot, I., Montel, V., Dickès-Coopman, A., Lesage, J., & Vieau, D. (2005a). Prenatal morphine exposure affects sympathoadrenal axis activity and serotonin metabolism in adult male rats both under basal conditions and after an ether inhalation stress. *Neuroscience Letters*, *381*(3), 211–216. https://doi.org/10.1016/j.neulet.2005.01.083

Laborie, C., Dutriez-Casteloot, I., Montel, V., Dickès-Coopman, A., Lesage, J., & Vieau, D. (2005b). Prenatal morphine exposure affects sympathoadrenal axis activity and serotonin metabolism in adult male rats both under basal conditions and after an ether inhalation stress. *Neuroscience Letters*, *381*(3), 211–216. https://doi.org/10.1016/j.neulet.2005.01.083

Lajtha, A., & Sershen, H. (2010). Nicotine: Alcohol reward interactions. In *Neurochemical Research* (Vol. 35, Issue 8, pp. 1248–1258). Springer. https://doi.org/10.1007/s11064-010-0181-8

Lamberts, J. T., Rosenthal, L. D., Jutkiewicz, E. M., & Traynor, J. R. (2018). Role of the guanine nucleotide binding protein, Gαo, in the development of morphine tolerance and dependence. *Psychopharmacology*, *235*(1), 71–82. https://doi.org/10.1007/s00213-017-4742-2

Langlois, L. D., & Nugent, F. S. (2017). Opiates and Plasticity in the Ventral Tegmental Area. In *ACS Chemical Neuroscience* (Vol. 8, Issue 9, pp. 1830–1838). American Chemical Society. https://doi.org/10.1021/acschemneuro.7b00281

Lasek, A. W., Janak, P. H., He, L., Whistler, J. L., & Heberlein, U. (2007). Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. *Genes, Brain and Behavior*, *6*(8), 728–735. https://doi.org/10.1111/j.1601-183X.2007.00303.x

Laurence Lalanne, Gulebru Ayranci, Brigitte, L. K., & Lutz, P. E. (2014). The kappa opioid receptor: From addiction to depression, and back. In *Frontiers in Psychiatry* (Vol. 5, Issue DEC). Frontiers Media S.A. https://doi.org/10.3389/fpsyt.2014.00170

Lazic, S. E. (2012). Modeling hippocampal neurogenesis across the lifespan in seven species. *Neurobiology of Aging*, *33*(8), 1664–1671. https://doi.org/10.1016/j.neurobiolaging.2011.03.008

Lebow, M. A., Schroeder, M., Tsoory, M., Holzman-Karniel, D., Mehta, D., Ben-Dor, S., Gil, S., Bradley, B., Smith, A. K., Jovanovic, T., Ressler, K. J., Binder, E. B., & Chen, A. (2019). Glucocorticoid-induced leucine zipper “quantifies” stressors and increases male susceptibility to PTSD. *Translational Psychiatry*, *9*(1). https://doi.org/10.1038/s41398-019-0509-3

Lee, H., Jang, M., Kim, W., & Noh, J. (2017). Differential effects of pair housing on voluntary nicotine consumption: a comparison between male and female adolescent rats. *Psychopharmacology*, *234*(16), 2463–2473. https://doi.org/10.1007/s00213-017-4636-3

Leeman, R. F., Nogueira, C., Wiers, R. W., Cousijn, J., Serafini, K., DeMartini, K. S., Bargh, J. A., & O’Malley, S. S. (2018). A Test of Multisession Automatic Action Tendency Retraining to Reduce Alcohol Consumption Among Young Adults in the Context of a Human Laboratory Paradigm. *Alcoholism: Clinical and Experimental Research*, *42*(4), 803–814. https://doi.org/10.1111/acer.13613

Lefevre, E. M., Pisansky, M. T., Toddes, C., Baruffaldi, F., Pravetoni, M., Tian, L., Kono, T. J. Y., & Rothwell, P. E. (2020). Interruption of continuous opioid exposure exacerbates drug-evoked adaptations in the mesolimbic dopamine system. *Neuropsychopharmacology*, *45*(11), 1781–1792. https://doi.org/10.1038/s41386-020-0643-x

Lehtonen, J., Shen, B., Vihinen, M., Casini, A., Scozzafava, A., Supuran, C. T., Parkkila, A. K., Saarnio, J., Kivelä, A. J., Waheed, A., Sly, W. S., & Parkkila, S. (2004). Characterization of CA XIII, a Novel Member of the Carbonic Anhydrase Isozyme Family. *Journal of Biological Chemistry*, *279*(4), 2719–2727. https://doi.org/10.1074/jbc.M308984200

Lejeune, C., Simmat-Durand, L., Gourarier, L., & Aubisson, S. (2006a). Prospective multicenter observational study of 260 infants born to 259 opiate-dependent mothers on methadone or high-dose buprenophine substitution the Groupe d'Etudes Grossesse et Addictions (GEGA) 1. *Drug and Alcohol Dependence*, *82*, 250–257. https://doi.org/10.1016/j.drugalcdep.2005.10.001

Lejeune, C., Simmat-Durand, L., Gourarier, L., & Aubisson, S. (2006b). Prospective multicenter observational study of 260 infants born to 259 opiate-dependent mothers on methadone or high-dose buprenophine substitution the Groupe d'Etudes Grossesse et Addictions (GEGA) 1. *Drug and Alcohol Dependence*, *82*, 250–257. https://doi.org/10.1016/j.drugalcdep.2005.10.001

Lemon, L. S., Caritis, S. N., Venkataramanan, R., Platt, R. W., & Bodnar, L. M. (2018). Methadone Versus Buprenorphine for Opioid Use Dependence and Risk of Neonatal Abstinence Syndrome. *Epidemiology*, *29*(2), 261–268. https://doi.org/10.1097/EDE.0000000000000780

Leonardo Jimenez Chavez, C., Bryant, C. D., Munn-chernoff, M. A., & Szumlinski, K. K. (2021). Selective inhibition of pde4b reduces binge drinking in two c57bl/6 substrains. *International Journal of Molecular Sciences*, *22*(11). https://doi.org/10.3390/ijms22115443

Lesage, J., Bernet, F., Montel, V., & Dupouy, J. P. (1996a). Effects of prenatal morphine on hypothalamic metabolism of neurotransmitters and gonadal and adrenal activities, during the early postnatal period in the rat. *Neurochemical Research*, *21*(6), 723–732. https://doi.org/10.1007/BF02527731

Lesage, J., Bernet, F., Montel, V., & Dupouy, J. P. (1996b). Effects of prenatal morphine on hypothalamic metabolism of neurotransmitters and gonadal and adrenal activities, during the early postnatal period in the rat. *Neurochemical Research*, *21*(6), 723–732. https://doi.org/10.1007/BF02527731

Lesage, J., Bernet, F., Montel, V., Dutriez-Casteloot, I., & Dupouy, J. P. (2000a). Influence of morphine treatment in pregnant rats on the mineralocorticoid activity of the adrenals in their neonates. *Life Sciences*, *66*(13), 1197–1211. https://doi.org/10.1016/S0024-3205(00)00425-2

Lesage, J., Bernet, F., Montel, V., Dutriez-Casteloot, I., & Dupouy, J. P. (2000b). Influence of morphine treatment in pregnant rats on the mineralocorticoid activity of the adrenals in their neonates. *Life Sciences*, *66*(13), 1197–1211. https://doi.org/10.1016/S0024-3205(00)00425-2

Leyenaar, J. K., Schaefer, A. P., Wasserman, J. R., Moen, E. L., O'Malley, A. J., & Goodman, D. C. (2021). Infant Mortality Associated with Prenatal Opioid Exposure. *JAMA Pediatrics*, *175*(7), 706–714. https://doi.org/10.1001/jamapediatrics.2020.6364

Li, J. J., Szkudlarek, H., Renard, J., Hudson, R., Rushlow, W., & Laviolette, S. R. (2018). Fear memory recall potentiates opiate reward sensitivity through dissociable dopamine D1 versus D4 receptor-dependent memory mechanisms in the prefrontal cortex. *Journal of Neuroscience*, *38*(19), 4543–4555. https://doi.org/10.1523/JNEUROSCI.3113-17.2018

Li, Y., Xia, B., Li, R., Yin, D., Wang, Y., & Liang, W. (2017). Expression of brain-derived neurotrophic factors, neurotrophin-3, and neurotrophin-4 in the nucleus accumbens during heroin dependency and withdrawal. *NeuroReport*, *28*(11), 654–660. https://doi.org/10.1097/WNR.0000000000000810

Lifschitz, M. H., Wilson, G. S., O'Brian Smith, E., & Desmond, M. M. (1985). Factors affecting head growth and intellectual function in children of drug addicts. *Pediatrics*, *75*(2), 269–274. https://doi.org/10.1542/peds.75.2.269

Lingle, S., Wyman, M. T., Kotrba, R., Teichroeb, L. J., & Romanow, C. A. (2012). What makes a cry a cry? A review of infant distress vocalizations. In *Current Zoology* (Vol. 58, Issue 5, pp. 698–726). https://doi.org/10.1093/czoolo/58.5.698

Linsenbardt, D. N., & Boehm, S. L. (2014). Alterations in the rate of binge ethanol consumption: Implications for preclinical studies in mice. *Addiction Biology*, *19*(5), 812–825. https://doi.org/10.1111/adb.12052

Lipari, R. N., & Park-Lee, E. (2020). *Key Substance Use and Mental Health Indicators in the United States: Results from the 2019 National Survey on Drug Use and Health*.

Liu, A., Dai, Y., Mendez, E. F., Hu, R., Fries, G. R., Najera, K. E., Jiang, S., Meyer, T. D., Stertz, L., Jia, P., Walss-Bass, C., & Zhao, Z. (2021). Genome-wide correlation of dna methylation and gene expression in postmortem brain tissues of opioid use disorder patients. *International Journal of Neuropsychopharmacology*, *24*(11), 879–891. https://doi.org/10.1093/ijnp/pyab043

Liu, T., Ivaturi, V., & Gobburu, J. (2019). Integrated Model to Describe Morphine Pharmacokinetics in Humans. *Journal of Clinical Pharmacology*, *59*(8), 1070–1077. https://doi.org/10.1002/jcph.1400

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12). https://doi.org/10.1186/s13059-014-0550-8

Lowrance, S. A., Ionadi, A., McKay, E., Douglas, X., & Johnson, J. D. (2016). Sympathetic nervous system contributes to enhanced corticosterone levels following chronic stress. *Psychoneuroendocrinology*, *68*, 163–170. https://doi.org/10.1016/j.psyneuen.2016.02.027

Luby, J. L., Baram, T. Z., Rogers, C. E., & Barch, D. M. (2020). Neurodevelopmental Optimization after Early-Life Adversity: Cross-Species Studies to Elucidate Sensitive Periods and Brain Mechanisms to Inform Early Intervention. In *Trends in Neurosciences* (Vol. 43, Issue 10, pp. 744–751). Elsevier Ltd. https://doi.org/10.1016/j.tins.2020.08.001

Lugo, R. A., & Kern, S. E. (2002). Clinical pharmacokinetics of morphine. In *Journal of Pain and Palliative Care Pharmacotherapy* (Vol. 16, Issue 4, pp. 5–18). https://doi.org/10.1080/J354v16n04_02

Lutz, P. E., & Kieffer, B. L. (2013). The multiple facets of opioid receptor function: Implications for addiction. In *Current Opinion in Neurobiology* (Vol. 23, Issue 4, pp. 473–479). https://doi.org/10.1016/j.conb.2013.02.005

MacArthur, C. J., & Trune, D. R. (2006). Mouse models of otitis media. In *Current Opinion in Otolaryngology and Head and Neck Surgery* (Vol. 14, Issue 5, pp. 341–346). Curr Opin Otolaryngol Head Neck Surg. https://doi.org/10.1097/01.moo.0000244193.97301.d7

Madras, B. K., Han, B., Compton, W. M., Jones, C. M., Lopez, E. I., & McCance-Katz, E. F. (2019). Associations of Parental Marijuana Use With Offspring Marijuana, Tobacco, and Alcohol Use and Opioid Misuse. *JAMA Network Open*, *2*(11), e1916015. https://doi.org/10.1001/jamanetworkopen.2019.16015

Magnan, J., & Tiberi, M. (1989). Evidence for the presence of μ- and κ- but not of δ-opioid sites in the human fetal brain. *Developmental Brain Research*, *45*(2), 275–281. https://doi.org/10.1016/0165-3806(89)90045-X

Maguire, D. J., Taylor, S., Armstrong, K., Shaffer-Hudkins, E., Germain, A. M., Brooks, S. S., Cline, G. J., & Clark, L. (2016). Long-term outcomes of infants with neonatal abstinence syndrome. *Neonatal Network*, *35*(5), 277–286. https://doi.org/10.1891/0730-0832.35.5.277

Malek, A., & R. Mattison, D. (2011). Drugs and Medicines in Pregnancy: The Placental Disposition of Opioids. *Current Pharmaceutical Biotechnology*, *12*(5), 797–803. https://doi.org/10.2174/138920111795470859

Malin, D. H., Lake, J. R., Carter, V. A., Cunningham, J. S., & Wilson, O. B. (1993). Naloxone precipitates nicotine abstinence syndrome in the rat. *Psychopharmacology*, *112*(2–3), 339–342. https://doi.org/10.1007/BF02244930

Margolis, E. B., Fields, H. L., Hjelmstad, G. O., & Mitchell, J. M. (2008). δ-opioid receptor expression in the ventral tegmental area protects against elevated alcohol consumption. *Journal of Neuroscience*, *28*(48), 12672–12681. https://doi.org/10.1523/JNEUROSCI.4569-08.2008

Margolis, E. B., Lock, H., Chefer, V. D., Shippenberg, T. S., Hjelmstad, G. O., & Fields, H. L. (2006). κ opioids selectivity control dopaminergic neurons projecting to the prefrontal cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(8), 2938–2942. https://doi.org/10.1073/pnas.0511159103

Marudhai, S., Patel, M., Valaiyaduppu Subas, S., Ghani, M. R., Busa, V., Dardeir, A., & Cancarevic, I. (2020). Long-term Opioids Linked to Hypogonadism and the Role of Testosterone Supplementation Therapy. *Cureus*, *12*(10). https://doi.org/10.7759/cureus.10813

Matthes, H. W. D., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dolie, P., Tzavara, E., Hanoune, J., Roques, B. P., & Kieffer, B. L. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature*, *383*(6603), 822–823. https://doi.org/10.1038/383819a0

Mayberry, H. L., Bavley, C. C., Karbalaei, R., Peterson, D. R., Bongiovanni, A. R., Ellis, A. S., Downey, S. H., Toussaint, A. B., & Wimmer, M. E. (2022). Transcriptomics in the nucleus accumbens shell reveal sex- and reinforcer-specific signatures associated with

morphine and sucrose craving. *Neuropsychopharmacology*, 1–12. https://doi.org/10.1038/s41386-022-01289-2

McBride, W. J., Chernet, E., McKinzie, D. L., Lumeng, L., & Li, T. K. (1998). Quantitative autoradiography of mu-opioid receptors in the CNS of alcohol-naive alcohol-preferring P and -nonpreferring NP rats. *Alcohol*, *16*(4), 317–323. https://doi.org/10.1016/S0741-8329(98)00021-4

McClellan Stine, M., Pfaff, D., Vandenbergh, D., & Cousino Klein, L. (2003). Maternal nicotine exposure increases nicotine preference in periadolescent male but not female C57Bl/6J mice. *Nicotine & Tobacco Research*, *5*(1), 117–124. https://doi.org/10.1080/1462220021000060446

McClung, C. A., Nestler, E. J., & Zachariou, V. (2005). Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *Journal of Neuroscience*, *25*(25), 6005–6015. https://doi.org/10.1523/JNEUROSCI.0062-05.2005

McLemore, G. L., Lewis, T., Jones, C. H., & Gauda, E. B. (2013). Novel pharmacotherapeutic strategies for treatment of opioid-induced neonatal abstinence syndrome. In *Seminars in Fetal and Neonatal Medicine* (Vol. 18, Issue 1, pp. 35–41). NIH Public Access. https://doi.org/10.1016/j.siny.2012.09.002

Meier, S. M., Trontti, K., Purves, K. L., Als, T. D., Grove, J., Laine, M., Pedersen, M. G., Bybjerg-Grauholm, J., Bækved-Hansen, M., Sokolowska, E., Mortensen, P. B., Hougaard, D. M., Werge, T., Nordentoft, M., Breen, G., Børglum, A. D., Eley, T. C., Hovatta, I., Mattheisen, M., & Mors, O. (2019). Genetic Variants Associated with Anxiety and Stress-Related Disorders: A Genome-Wide Association Study and Mouse-Model Study. *JAMA Psychiatry*, *76*(9), 924–932. https://doi.org/10.1001/jamapsychiatry.2019.1119

Merhar, S. L., Parikh, N. A., Braimah, A., Poindexter, B. B., Tkach, J., & Kline-Fath, B. (2019). White matter injury and structural anomalies in infants with prenatal opioid exposure. *American Journal of Neuroradiology*, *40*(12), 2161–2165. https://doi.org/10.3174/ajnr.A6282

Minnes, S., Lang, A., & Singer, L. (2011). Prenatal tobacco, marijuana, stimulant, and opiate exposure: outcomes and practice implications. In *Addiction science & clinical practice* (Vol. 6, Issue 1, pp. 57–70).

Mitchell, J. M., Margolis, E. B., Coker, A. R., & Fields, H. L. (2012). Alcohol self-administration, anxiety, and cortisol levels predict changes in delta opioid receptor function in the ventral tegmental area. *Behavioral Neuroscience*, *126*(4), 515–522. https://doi.org/10.1037/a0029027

Mitchell, J. M., O'Neil, J. P., Janabi, M., Marks, S. M., Jagust, W. J., & Fields, H. L. (2012). Alcohol consumption induces endogenous opioid release in the human orbitofrontal cortex and nucleus accumbens. *Science Translational Medicine*, *4*(116), 116ra6-116ra6. https://doi.org/10.1126/scitranslmed.3002902

Mokrani, M. C., Duval, F., Crocq, M. A., Bailey, P., & Macher, J. P. (1997). HPA axis dysfunction in depression: Correlation with monoamine system abnormalities. *Psychoneuroendocrinology*, *22*(SUPPL. 1). https://doi.org/10.1016/S0306-4530(97)00012-7

Moles, A., Kieffer, B. L., & D'Amato, F. R. (2004). Deficit in attachment behavior in mice lacking the μ-opioid receptor gene. *Science*, *304*(5679), 1983–1986. https://doi.org/10.1126/science.1095943

Moles, A., Sarli, C., Bartolomucci, A., & D'Amato, F. R. (2008). Interaction with stressed mothers affects corticosterone levels in pups after reunion and impairs the response to dexamethasone in adult mice. *Psychoneuroendocrinology*, *33*(4), 462–470. https://doi.org/10.1016/j.psyneuen.2008.01.003

Möller, C., Wolf, F., van Dijk, R. M., Di Liberto, V., Russmann, V., Keck, M., Palme, R., Hellweg, R., Gass, P., Otzdorff, C., & Potschka, H. (2018). Toward evidence-based severity assessment in rat models with repeated seizures: I. Electrical kindling. *Epilepsia*, *59*(4), 765–777. https://doi.org/10.1111/epi.14028

Montandon, G., & Slutsky, A. S. (2019). *Solving the Opioid Crisis Respiratory Depression by Opioids as Critical End Point*. https://doi.org/10.1016/j.chest.2019.05.015

Mooney-Leber, S. M., & Gould, T. J. (2018). The long-term cognitive consequences of adolescent exposure to recreational drugs of abuse. *Learning and Memory*, *25*(9), 481–491. https://doi.org/10.1101/lm.046672.117

Moore, J. N., Gastonguay, M. R., Ng, C. M., Adeniyi-Jones, S. C., Moody, D. E., Fang, W. B., Ehrlich, M. E., & Kraft, W. K. (2018). The Pharmacokinetics and Pharmacodynamics of Buprenorphine in Neonatal Abstinence Syndrome. *Clinical Pharmacology and Therapeutics*, *103*(6), 1029–1037. https://doi.org/10.1002/cpt.1064

Moore, T. M., White, L. K., Barzilay, R., Calkins, M. E., Jones, J. D., Young, J. F., Gur, R. C., & Gur, R. E. (2020). Development of a scale battery for rapid assessment of risk and resilience. *Psychiatry Research*, *288*. https://doi.org/10.1016/j.psychres.2020.112996

Morales, I., Rodríguez-Borillo, O., Font, L., & Pastor, R. (2020). Effects of naltrexone on alcohol, sucrose, and saccharin binge-like drinking in C57BL/6J mice: a study with a multiple bottle choice procedure. *Behavioural Pharmacology*, *31*(2), 256–271. https://doi.org/10.1097/FBP.0000000000000553

Moultrie, F., Slater, R., & Hartley, C. (2017). Improving the treatment of infant pain. In *Current Opinion in Supportive and Palliative Care* (Vol. 11, Issue 2, pp. 112–117). Lippincott Williams and Wilkins. https://doi.org/10.1097/SPC.0000000000000270

Muldoon, P. P., Jackson, K. J., Perez, E., Harenza, J. L., Molas, S., Rais, B., Anwar, H., Zaveri, N. T., Maldonado, R., Maskos, U., McIntosh, J. M., Dierssen, M., Miles, M. F., Chen, X., de Biasi, M., & Damaj, M. I. (2014). The α3β4* nicotinic ACh receptor subtype mediates physical dependence to morphine: Mouse and human studies. *British Journal of Pharmacology*, *171*(16), 3845–3857. https://doi.org/10.1111/bph.12741

Nakhjiri, E., Saboory, E., Roshan-Milani, S., Rasmi, Y., & Khalafkhani, D. (2017). Effect of prenatal restraint stress and morphine co-administration on plasma vasopressin concentration and anxiety behaviors in adult rat offspring. *Stress*, *20*(2), 205–211. https://doi.org/10.1080/10253890.2017.1306053

Nasiraei-Moghadam, S., Sherafat, M. A., Safari, M. S., Moradi, F., Ahmadiani, A., & Dargahi, L. (2013). Reversal of prenatal morphine exposure-induced memory deficit in male but not female rats. *Journal of Molecular Neuroscience*, *50*(1), 58–69. https://doi.org/10.1007/s12031-012-9860-z

Navandar, M., Martín-García, E., Maldonado, R., Lutz, B., Gerber, S., & Ruiz de Azua, I. (2021). Transcriptional signatures in prefrontal cortex confer vulnerability versus resilience to food and cocaine addiction-like behavior. *Scientific Reports*, *11*(1), 9076. https://doi.org/10.1038/s41598-021-88363-9

Nesic, J., Rusted, J., Duka, T., & Jackson, A. (2011). Degree of dependence influences the effect of smoking on cognitive flexibility. *Pharmacology Biochemistry and Behavior*, *98*(3), 376–384. https://doi.org/10.1016/j.pbb.2011.01.015

Neugebauer, N. M., Einstein, E. B., Lopez, M. B., McClure-Begley, T. D., Mineur, Y. S., & Picciotto, M. R. (2013). Morphine dependence and withdrawal induced changes in cholinergic signaling. *Pharmacology Biochemistry and Behavior*, *109*, 77–83. https://doi.org/10.1016/j.pbb.2013.04.015

Nguyen, A. T., Armstrong, E. A., & Yager, J. Y. (2017). Neurodevelopmental reflex testing in neonatal rat pups. *Journal of Visualized Experiments*, *2017*(122), 55261. https://doi.org/10.3791/55261

Nickel, B. (1987). The antinociceptive activity of flupirtine: A structurally new analgesic. *Postgraduate Medical Journal*, *63*(SUPPL. 3), 19–28.

Nicolas, C., Zlbnik, N. E., Farokhnia, M., Lggio, L., Ikmoto, S., & Shaham, Y. (2022). Sex Differences in Opioid and Psychostimulant Craving and Relapse: A Critical ReviewS. *Pharmacological Reviews*, *74*(1), 119–140. https://doi.org/10.1124/pharmrev.121.000367

Nixon, R. (1971). Special Message to the Congress on Drug Abuse Prevention and Control. *Public Papers of the Presidents of the United States*, 739– 49.

Njung'e, K., & Handley, S. L. (1991). Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *British Journal of Pharmacology*, *104*(1), 105–112. https://doi.org/10.1111/j.1476-5381.1991.tb12392.x

Nygaard, E., Moe, V., Slinning, K., & Walhovd, K. B. (2015). Longitudinal cognitive development of children born to mothers with opioid and polysubstance use. *Pediatric Research*, *78*(3), 330–335. https://doi.org/10.1038/pr.2015.95

Nygaard, E., Slinning, K., Moe, V., Fjell, A., & Walhovd, K. B. (2020a). Mental health in youth prenatally exposed to opioids and poly-drugs and raised in permanent

foster/adoptive homes: A prospective longitudinal study. *Early Human Development*, *140*. https://doi.org/10.1016/j.earlhumdev.2019.104910

Nygaard, E., Slinning, K., Moe, V., Fjell, A., & Walhovd, K. B. (2020b). Mental health in youth prenatally exposed to opioids and poly-drugs and raised in permanent foster/adoptive homes: A prospective longitudinal study. *Early Human Development*, *140*, 104910. https://doi.org/10.1016/j.earlhumdev.2019.104910

Nygaard, E., Slinning, K., Moe, V., & Walhovd, K. B. (2017). Cognitive function of youths born to mothers with opioid and poly-substance abuse problems during pregnancy. *Child Neuropsychology*, *23*(2), 159–187. https://doi.org/10.1080/09297049.2015.1092509

O'Neal, T. J., Nooney, M. N., Thien, K., & Ferguson, S. M. (2020). Chemogenetic modulation of accumbens direct or indirect pathways bidirectionally alters reinstatement of heroin-seeking in high- but not low-risk rats. *Neuropsychopharmacology*, *45*(8), 1251–1262. https://doi.org/10.1038/s41386-019-0571-9

Ornoy, A., & Koren, G. (2019). Ssris and snris (Sri) in pregnancy: Effects on the course of pregnancy and the offspring: How far are we from having all the answers? In *International Journal of Molecular Sciences* (Vol. 20, Issue 10). MDPI AG. https://doi.org/10.3390/ijms20102370

Ornoy, A., Michailevskaya, V., Lukashov, I., Bar-Hamburger, R., & Harel, S. (1996). The developmental outcome of children born to heroin-dependent mothers, raised at home or adopted. *Child Abuse and Neglect*, *20*(5), 385–396. https://doi.org/10.1016/0145-2134(96)00014-2

Ortega, L. A., Tracy, B. A., Gould, T. J., & Parikh, V. (2013). Effects of chronic low- and high-dose nicotine on cognitive flexibility in C57BL/6J mice. *Behavioural Brain Research*, *238*(1), 134–145. https://doi.org/10.1016/j.bbr.2012.10.032

Oslin, D. W., Berrettini, W. H., & O'Brien, C. P. (2006). Targeting treatments for alcohol dependence: The pharmacogenetics of naltrexone. In *Addiction Biology* (Vol. 11, Issues 3–4, pp. 397–403). John Wiley & Sons, Ltd. https://doi.org/10.1111/j.1369-1600.2006.00036.x

Ostrander, M. M., Ulrich-Lai, Y. M., Choi, D. C., Richtand, N. M., & Herman, J. P. (2006). Hypoactivity of the hypothalamo-pituitary-adrenocortical axis during recovery from chronic variable stress. *Endocrinology*, *147*(4), 2008–2017. https://doi.org/10.1210/en.2005-1041

Ostrea, E. M., Ostrea, A. R., & Simpson, P. M. (1997). Mortality within the first 2 years in infants exposed to cocaine, opiate, or cannabinoid during gestation. *Pediatrics*, *100*(1), 79–83. https://doi.org/10.1542/peds.100.1.79

Pacifici, G. M. (2016). Metabolism and pharmacokinetics of morphine in neonates: A review. In *Clinics* (Vol. 71, Issue 8, pp. 474–480). https://doi.org/10.6061/clinics/2016(08)11

Panlilio, L. V., & Goldberg, S. R. (2007). Self-administration of drugs in animals and humans as a model and an investigative tool. In *Addiction* (Vol. 102, Issue 12, pp. 1863–1870). NIH Public Access. https://doi.org/10.1111/j.1360-0443.2007.02011.x

Papaleo, F., & Contarino, A. (2006). Gender- and morphine dose-linked expression of spontaneous somatic opiate withdrawal in mice. *Behavioural Brain Research*, *170*(1), 110–118. https://doi.org/10.1016/j.bbr.2006.02.009

Parga, J. J., Lewin, S., Lewis, J., Montoya-Williams, D., Alwan, A., Shaul, B., Han, C., Bookheimer, S. Y., Eyer, S., Dapretto, M., Zeltzer, L., Dunlap, L., Nookala, U., Sun, D., Dang, B. H., & Anderson, A. E. (2020). Defining and distinguishing infant behavioral states using acoustic cry analysis: is colic painful? *Pediatric Research*, *87*(3), 576–580. https://doi.org/10.1038/s41390-019-0592-4

Patel, I., Walter, L. A., & Li, L. (2021). Opioid overdose crises during the COVID-19 pandemic: implication of health disparities. *Harm Reduction Journal*, *18*(1). https://doi.org/10.1186/s12954-021-00534-z

Pathan, H., & Williams, J. (2012). Basic opioid pharmacology: an update. *British Journal of Pain*, *6*(1), 11–16. https://doi.org/10.1177/2049463712438493

Patrick, S. W., Schiff, D. M., Ryan, S. A., Quigley, J., Gonzalez, P. K., & Walker, L. R. (2017a). A public health response to opioid use in pregnancy. *Pediatrics*, *139*(3). https://doi.org/10.1542/peds.2016-4070

Patrick, S. W., Schiff, D. M., Ryan, S. A., Quigley, J., Gonzalez, P. K., & Walker, L. R. (2017b). A public health response to opioid use in pregnancy. *Pediatrics*, *139*(3). https://doi.org/10.1542/peds.2016-4070

Pearse, D. D., & Hughes, Z. A. (2016). PDE4B as a microglia target to reduce neuroinflammation. *Glia*, *64*(10), 1698–1709. https://doi.org/10.1002/glia.22986

Peleg-Oren, N., & Teichman, M. (2006). Young children of parents with Substance Use Disorders (SUD): A review of the literature and implications for social work practice. In *Journal of Social Work Practice in the Addictions* (Vol. 6, Issues 1–2, pp. 49–61). https://doi.org/10.1300/J160v06n01_03

Pennington, B., Collins, B., Leigh, S., Martin, A. P., Owen, L., Fischer, A., Sumnall, H., & Bates, G. (2018). The cost-effectiveness of seven behavioural interventions to prevent drug misuse in vulnerable populations. *International Journal of Drug Policy*, *57*, 42–50. https://doi.org/10.1016/j.drugpo.2018.03.028

Pereira de Souza Goldim, M., Della Giustina, A., Mathias, K., de Oliveira Junior, A., Fileti, M. E., De Carli, R., Zarbato, G., Garbossa, L., da Rosa, N., Oliveira, J., Vieira, A., Generoso, J., Oliveira, B. H., Ferreira, N., Palandi, J., Bobinski, F., Martins, D. F., Fortunato, J., Barichello, T., … Petronilho, F. (2020). Sickness Behavior Score Is Associated with Neuroinflammation and Late Behavioral Changes in Polymicrobial Sepsis Animal Model. *Inflammation*, *43*(3), 1019–1034. https://doi.org/10.1007/s10753-020-01187-z

Perez, E. E., & De Biasi, M. (2015). Assessment of affective and somatic signs of ethanol withdrawal in C57BL/6J mice using a short-term ethanol treatment. *Alcohol (Fayetteville, N.Y.)*, *49*(3), 237–243. https://doi.org/10.1016/j.alcohol.2015.02.003

Perez, E., Quijano-Cardé, N., & De Biasi, M. (2015). Nicotinic Mechanisms Modulate Ethanol Withdrawal and Modify Time Course and Symptoms Severity of Simultaneous Withdrawal from Alcohol and Nicotine. *Neuropsychopharmacology*, *40*, 2327–2336. https://doi.org/10.1038/npp.2015.80

Pfizer. (n.d.). *morphine sulfate injection, USP VIAL Use in Specific Populations | Pfizer Medical Information - US*. Retrieved August 14, 2022, from https://www.pfizermedicalinformation.com/en-us/morphine-0/population-use

Piazza, P. V., & Le Moal, M. (1997). Glucocorticoids as a biological substrate of reward: Physiological and pathophysiological implications. In *Brain Research Reviews* (Vol. 25, Issue 3, pp. 359–372). Brain Res Brain Res Rev. https://doi.org/10.1016/S0165-0173(97)00025-8

Piccotti, L., Voigtman, B., Vongsa, R., Nellhaus, E. M., Rodriguez, K. J., Davies, T. H., & Quirk, S. (2019). Neonatal Opioid Withdrawal Syndrome: A Developmental Care Approach. In *Neonatal network : NN* (Vol. 38, Issue 3, pp. 160–169). NLM (Medline). https://doi.org/10.1891/0730-0832.38.3.160

Pinelli, A., & Trivulzio, S. (1997). Quantitative evaluation of opioid withdrawal signs in rats repeatedly treated with morphine and injected with naloxone, in the absence or presence of the antiabstinence agent clonidine. *Journal of Pharmacological and Toxicological Methods*, *38*(3), 117–131. https://doi.org/10.1016/S1056-8719(97)00050-6

Piskunov, A., Stepanichev, M., Tishkina, A., Novikova, M., Levshina, I., & Gulyaeva, N. (n.d.). *Chronic combined stress induces selective and long-lasting inflammatory response evoked by changes in corticosterone accumulation and signaling in rat hippocampus*. https://doi.org/10.1007/s11011-015-9785-7

Przewłoka, B., Lason, W., & Przewłocki, R. (1994). Repeated ethanol differently affects opioid peptide biosynthesis in the rat pituitary. *Neuroendocrinology*, *60*(3), 331–336. https://doi.org/10.1159/000126766

Qin, D. D., Rizak, J., Feng, X. L., Yang, S. C., Lü, L. B., Pan, L., Yin, Y., & Hu, X. T. (2016). Prolonged secretion of cortisol as a possible mechanism underlying stress and depressive behaviour. *Scientific Reports*, *6*. https://doi.org/10.1038/srep30187

Qu, C. L., Huo, F. Q., Huang, F. S., & Tang, J. S. (2015). Activation of mu-opioid receptors in the ventrolateral orbital cortex inhibits the GABAergic miniature inhibitory postsynaptic currents in rats. *Neuroscience Letters*, *592*, 64–69. https://doi.org/10.1016/j.neulet.2015.02.045

Quijano Cardé, N. A., & De Biasi, M. (2022). Behavioral characterization of withdrawal following chronic voluntary ethanol consumption via intermittent two-bottle choice points to different susceptibility categories. *Alcoholism: Clinical and Experimental Research*. https://doi.org/10.1111/acer.14785

Quijano Cardé, N. A., Perez, E. E., Feinn, R., Kranzler, H. R., & De Biasi, M. (2021). Antagonism of GluK1-containing kainate receptors reduces ethanol consumption by modulating ethanol reward and withdrawal. *Neuropharmacology*, *199*, 108783. https://doi.org/10.1016/j.neuropharm.2021.108783

Quijano Cardé, N. A., Shaw, J., Carter, C., Kim, S., Stitzel, J. A., Venkatesh, S. K., Ramchandani, V. A., & De Biasi, M. (2022). Mutation of the α5 nicotinic acetylcholine receptor subunit increases ethanol and nicotine consumption in adolescence and impacts adult drug consumption. *Neuropharmacology*, *216*, 109170. https://doi.org/10.1016/j.neuropharm.2022.109170

Racz, I., Schürmann, B., Karpushova, A., Reuter, M., Cichon, S., Montag, C., Fürst, R., Schütz, C., Franke, P. E., Strohmaier, J., Wienker, T. F., Terenius, L., Ösby, U., Gunnar, A., Maier, W., Bilkei-Gorzó, A., Nöthen, M., & Zimmer, A. (2008). The Opioid Peptides Enkephalin and β-Endorphin in Alcohol Dependence. *Biological Psychiatry*, *64*(11), 989–997. https://doi.org/10.1016/j.biopsych.2008.05.008

Radhakrishna, U., Nath, S. K., Vishweswaraiah, S., Uppala, L. V., Forray, A., Muvvala, S. B., Mishra, N. K., Southekal, S., Guda, C., Govindamangalam, H., Vargas, D., Gardella, W. G., Crist, R. C., Berrettini, W. H., Metpally, R. P., & Bahado-Singh, R. O. (2021). Maternal opioid use disorder: Placental transcriptome analysis for neonatal opioid withdrawal syndrome. *Genomics*, *113*(6), 3610–3617. https://doi.org/10.1016/j.ygeno.2021.08.001

Radhakrishnan, R., Vishnubhotla, R. V., Guckien, Z., Zhao, Y., Sokol, G. M., Haas, D. M., & Sadhasivam, S. (2022). Thalamocortical functional connectivity in infants with prenatal opioid exposure correlates with severity of neonatal opioid withdrawal syndrome. *Neuroradiology*, *1*, 3. https://doi.org/10.1007/s00234-022-02939-4

Radke, A. K., Sneddon, E. A., & Monroe, S. C. (2021). Studying Sex Differences in Rodent Models of Addictive Behavior. *Current Protocols*, *1*(4). https://doi.org/10.1002/cpz1.119

Raffa, R. B., Friderichs, E., Reimann, W., Shank, R. P., Codd, E. E., & Vaught, J. L. (1992). Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an “atypical” opioid analgesic. *Journal of Pharmacology and Experimental Therapeutics*, *260*(1), 275–285.

Ramsey, N. F., Niesink, R. J. M., & Van Ree, J. M. (1993a). Prenatal exposure to morphine enhances cocaine and heroin self-administration in drug-naive rats. *Drug and Alcohol Dependence*, *33*(1), 41–51. https://doi.org/10.1016/0376-8716(93)90032-L

Ramsey, N. F., Niesink, R. J. M., & Van Ree, J. M. (1993b). Prenatal exposure to morphine enhances cocaine and heroin self-administration in drug-naive rats. *Drug and Alcohol Dependence*, *33*(1), 41–51. https://doi.org/10.1016/0376-8716(93)90032-L

Ray, L. A., MacKillop, J., Leggio, L., Morgan, M., & Hutchison, K. E. (2009). Effects of naltrexone on cortisol levels in heavy drinkers. *Pharmacology Biochemistry and Behavior*, *91*(4), 489–494. https://doi.org/10.1016/j.pbb.2008.09.004

Ray, S. B., & Wadhwa, S. (1999). Mu opioid receptors in developing human spinal cord. *Journal of Anatomy*, *195*(1), 11–18. https://doi.org/10.1017/S0021878299004963

Read, J. P., Beattie, M., Chamberlain, R., & Merrill, J. E. (2008). Beyond the "Binge" threshold: Heavy drinking patterns and their association with alcohol involvement indices in college students. *Addictive Behaviors*, *33*(2), 225–234. https://doi.org/10.1016/j.addbeh.2007.09.001

Reddy, U. M., Davis, J. M., Ren, Z., & Greene, M. F. (2017). Opioid use in pregnancy, neonatal abstinence syndrome, and childhood outcomes. In *Obstetrics and Gynecology* (Vol. 130, Issue 1, pp. 10–28). Lippincott Williams and Wilkins. https://doi.org/10.1097/AOG.0000000000002054

Ren, M., & Lotfipour, S. (2019). Nicotine gateway effects on adolescent substance use. In *Western Journal of Emergency Medicine* (Vol. 20, Issue 5, pp. 696–709). eScholarship. https://doi.org/10.5811/westjem.2019.7.41661

Rensing, N., Moy, B., Friedman, J. L., Galindo, R., & Wong, M. (2018). Longitudinal analysis of developmental changes in electroencephalography patterns and sleep-wake states of the neonatal mouse. *PLOS ONE*, *13*(11), e0207031. https://doi.org/10.1371/journal.pone.0207031

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., & Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology and Behavior*, *84*(1), 53–63. https://doi.org/10.1016/j.physbeh.2004.10.007

Rhodes, J. S., Ford, M. M., Yu, C.-H., Brown, L. L., Finn, D. A., Garland, T., & Crabbe, J. C. (2007). Mouse inbred strain differences in ethanol drinking to intoxication. *Genes, Brain and Behavior*, *6*(1), 1–18. https://doi.org/10.1111/j.1601-183X.2006.00210.x

Riala, K., Hakko, H., Isohanni, M., Järvelin, M.-R., & Räsänen, P. (2004). Teenage smoking and substance use as predictors of severe alcohol problems in late adolescence and in young adulthood. *Journal of Adolescent Health*, *35*(3), 245–254. https://doi.org/10.1016/j.jadohealth.2003.08.016

Rice, D., & Barone, S. (2000). Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environmental Health Perspectives*, *108*(SUPPL. 3), 511–533. https://doi.org/10.1289/ehp.00108s3511

Richard, S., & Flamant, F. (2018). Regulation of T3 availability in the developing brain: The mouse genetics contribution. In *Frontiers in Endocrinology* (Vol. 9, Issue MAY, p. 1). Frontiers Media S.A. https://doi.org/10.3389/fendo.2018.00265

Riley, M. A., & Vathy, I. (2006). Mid- to late gestational morphine exposure does not alter the rewarding properties of morphine in adult male rats. *Neuropharmacology*, *51*(2), 295–304. https://doi.org/10.1016/j.neuropharm.2006.03.022

Rimanóczy, Á., Šlamberová, R., Bar, N., & Vathy, I. (2006). Morphine exposure prevents up-regulation of MR and GR binding sites in the brain of adult male and female rats due to prenatal stress. *International Journal of Developmental Neuroscience*, *24*(4), 241–248. https://doi.org/10.1016/j.ijdevneu.2006.04.001

Rimanóczy, Á., Ŝlamberová, R., Riley, M. A., & Vathy, I. (2003). Adrenocorticotropin stress response but not glucocorticoid-negative feedback is altered by prenatal morphine exposure in adult male rats. *Neuroendocrinology*, *78*(6), 312–320. https://doi.org/10.1159/000074884

Rimanoczy, A., & Vathy, I. (1995). Prenatal exposure to morphine alters brain μ opioid receptor characteristics in rats. *Brain Research*, *690*(2), 245–248. https://doi.org/10.1016/0006-8993(95)00638-7

Rius, R. A., Barg, J., Bem, W. T., Coscia, C. J., & Loh, Y. P. (1991). The prenatal developmental profile of expression of opioid peptides and receptors in the mouse brain. *Developmental Brain Research*, *58*(2), 237–241. https://doi.org/10.1016/0165-3806(91)90010-G

Robinson, S. E. (2000). Effect of prenatal opioid exposure on cholinergic development. *Journal of Biomedical Science*, *7*(3), 253–257. https://doi.org/10.1007/BF02255474

Roche, D. J. O., Childs, E., Epstein, A. M., & King, A. C. (2010). Acute HPA axis response to naltrexone differs in female vs. male smokers. *Psychoneuroendocrinology*, *35*(4), 596–606. https://doi.org/10.1016/j.psyneuen.2009.09.016

Rockman, G. E., Hall, A., & Glavin, G. B. (1986). Effects of restraint stress on voluntary ethanol intake and ulcer proliferation in rats. *Pharmacology, Biochemistry and Behavior*, *25*(5), 1083–1087. https://doi.org/10.1016/0091-3057(86)90089-4

Rodgers, A. B., Morgan, C. P., Bronson, S. L., Revello, S., & Bale, T. L. (2013). Paternal stress exposure alters sperm MicroRNA content and reprograms offspring HPA stress axis regulation. *Journal of Neuroscience*, *33*(21), 9003–9012. https://doi.org/10.1523/JNEUROSCI.0914-13.2013

Rohbani, K., Sabzevari, S., Sadat-Shirazi, M. S., Nouri Zadeh-Tehrani, S., Ashabi, G., Khalifeh, S., Ale-Ebrahim, M., & Zarrindast, M. R. (2019). Parental morphine exposure affects repetitive grooming actions and marble burying behavior in the offspring: Potential relevance for obsessive-compulsive like behavior. *European Journal of Pharmacology*, *865*. https://doi.org/10.1016/j.ejphar.2019.172757

Rola, R., Jarkiewicz, M., & Szulczyk, P. (2008). *Modulation of Ca2+ channel current by mu opioid receptors in prefrontal cortex pyramidal neurons in rats - PubMed*. https://pubmed-ncbi-nlm-nih-gov.proxy.library.upenn.edu/18389010/

Rose, A. K., Hobbs, M., Klipp, L., Bell, S., Edwards, K., O'Hara, P., & Drummond, C. (2010). Monitoring drinking behaviour and motivation to drink over successive doses of alcohol. *Behavioural Pharmacology*, *21*(8), 710–718. https://doi.org/10.1097/FBP.0b013e32833fa72b

Rosen, L. G., Sun, N., Rushlow, W., & Laviolette, S. R. (2015). Molecular and neuronal plasticity mechanisms in the amygdala-prefrontal cortical circuit: Implications for opiate addiction memory formation. In *Frontiers in Neuroscience* (Vol. 9, Issue NOV, p. 399). Frontiers Research Foundation. https://doi.org/10.3389/fnins.2015.00399

Rosenblum, A., Marsch, L. A., Joseph, H., & Portenoy, R. K. (2008). Opioids and the Treatment of Chronic Pain: Controversies, Current Status, and Future Directions. *Experimental and Clinical Psychopharmacology*, *16*(5), 405–416. https://doi.org/10.1037/a0013628

Ross, E. J., Graham, D. L., Money, K. M., & Stanwood, G. D. (2015). Developmental consequences of fetal exposure to drugs: What we know and what we still must learn. In *Neuropsychopharmacology* (Vol. 40, Issue 1, pp. 61–87). https://doi.org/10.1038/npp.2014.147

Rozisky, J. R., Scarabelot, V. L., Oliveira, C. de, Macedo, I. C. de, Deitos, A., Laste, G., Caumo, W., & Torres, I. L. S. (2016). Melatonin as a potential counter-effect of hyperalgesia induced by neonatal morphine exposure. *Neuroscience Letters*, *633*, 77–81. https://doi.org/10.1016/j.neulet.2016.08.027

Ruffolo, J., Frie, J. A., Thorpe, H. H. A., Talhat, M. A., & Khokhar, J. Y. (2021). Alcohol and Vaporized Nicotine Co-exposure During Adolescence Contribute Differentially to Sex-Specific Behavioral Effects in Adulthood. *Nicotine & Tobacco Research*, *24*(8). https://doi.org/10.1093/ntr/ntab250

Russell, V. A. (2011). Overview of animal models of attention deficit hyperactivity disorder (ADHD). *Current Protocols in Neuroscience*, *Chapter 9*(SUPPL.54). https://doi.org/10.1002/0471142301.ns0935s54

Sabzevari, S., Rohbani, K., Sadat-Shirazi, M. S., Babhadi-Ashar, N., Shakeri, A., Ashabi, G., Khalifeh, S., Ale-Ebrahim, M., & Zarrindast, M. R. (2019). Morphine exposure before conception affects anxiety-like behavior and CRF level (in the CSF and plasma) in the adult male offspring. *Brain Research Bulletin*, *144*, 122–131. https://doi.org/10.1016/j.brainresbull.2018.11.022

Sadat-Shirazi, M. S., Karimi, F., Kaka, G., Ashabi, G., Ahmadi, I., Akbarabadi, A., Toolee, H., Vousooghi, N., & Zarrindast, M. R. (2019). Parental morphine exposure enhances morphine (but not methamphetamine) preference and increases monoamine oxidase-B level in the nucleus accumbens. *Behavioural Pharmacology*, *30*(5), 435–445. https://doi.org/10.1097/FBP.0000000000000465

Salas, R., Main, A., Gangitano, D. A., Zimmerman, G., Ben-Ari, S., Soreq, H., & De Biasi, M. (2008). Nicotine relieves anxiogenic-like behavior in mice that overexpress the read-through variant of acetylcholinesterase but not in wild-type mice. *Molecular Pharmacology*, *74*(6), 1641–1648. https://doi.org/10.1124/mol.108.048454

Salas, R., Main, A., Gangitano, D., & De Biasi, M. (2007). Decreased withdrawal symptoms but normal tolerance to nicotine in mice null for the α7 nicotinic acetylcholine receptor subunit. *Neuropharmacology*, *53*(7), 863–869. https://doi.org/10.1016/j.neuropharm.2007.08.017

Salas, R., Pieri, F., & De Biasi, M. (2004). Decreased signs of nicotine withdrawal in mice null for the β4 nicotinic acetylcholine receptor subunit. *Journal of Neuroscience*, *24*(45), 10035–10039. https://doi.org/10.1523/JNEUROSCI.1939-04.2004

Salas, R., Pieri, F., Fung, B., Dani, J. A., & De Biasi, M. (2003). Altered anxiety-related responses in mutant mice lacking the β4 subunit of the nicotinic receptor. *Journal of Neuroscience*, *23*(15), 6255–6263. https://doi.org/10.1523/jneurosci.23-15-06255.2003

Salas, R., Sturm, R., Boulter, J., & De Biasi, M. (2009). Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. *Journal of Neuroscience*, *29*(10), 3014–3018. https://doi.org/10.1523/JNEUROSCI.4934-08.2009

Salihu, H. M., Salinas, A., Medina, I., Krishnaswami, J., & Aliyu, M. H. (2019). Biopsychosocial determinants of opioid use disorder (OUD) and implications for maternal and child health research: A scoping review. In *Journal of Opioid Management* (Vol. 15, Issue 1, pp. 77–91). Weston Medical Publishing. https://doi.org/10.5055/jom.2019.0488

Salling, M. C., Skelly, M. J., Avegno, E., Regan, S., Zeric, T., Nichols, E., & Harrison, N. L. (2018). Alcohol consumption during adolescence in a mouse model of binge drinking alters the intrinsic excitability and function of the prefrontal cortex through a reduction in the hyperpolarization-activated cation current. *Journal of Neuroscience*, *38*(27), 6207–6222. https://doi.org/10.1523/JNEUROSCI.0550-18.2018

Sampath, D., White, A. M., & Raol, Y. H. (2014). Characterization of neonatal seizures in an animal model of hypoxic-ischemic encephalopathy. *Epilepsia*, *55*(7), 985–993. https://doi.org/10.1111/epi.12646

Santangeli, O., Lehtikuja, H., Palomäki, E., Wigren, H. K., Paunio, T., & Porkka-Heiskanen, T. (2016). Sleep and behavior in cross-fostering rats: Developmental and sex aspects. *Sleep*, *39*(12), 2211–2221. https://doi.org/10.5665/sleep.6328

Sarfi, M., Eikemo, M., Welle-Strand, G. K., Muller, A. E., & Lehmann, S. (2021). Mental health and use of health care services in opioid-exposed school-aged children compared to foster children. *European Child and Adolescent Psychiatry*, *1*, 3. https://doi.org/10.1007/s00787-021-01728-3

Sarkaki, A., Assaei, R., Motamedi, F., Badavi, M., & Pajouhi, N. (2008). Effect of parental morphine addiction on hippocampal long-term potentiation in rats offspring. *Behavioural Brain Research*, *186*(1), 72–77. https://doi.org/10.1016/j.bbr.2007.07.041

Sarkar, D. K. (2015). Fetal alcohol exposure increases susceptibility to carcinogenesis and promotes tumor progression in prostate gland. In *Advances in Experimental Medicine and Biology* (Vol. 815, pp. 389–402). Springer New York LLC. https://doi.org/10.1007/978-3-319-09614-8_23

Sarkar, D. K., Kuhn, P., Marano, J., Chen, C., & Boyadjieva, N. (2007). Alcohol exposure during the developmental period induces α-endorphin neuronal death and causes alteration in the opioid control of stress axis function. *Endocrinology*, *148*(6), 2828–2834. https://doi.org/10.1210/en.2006-1606

Sasamori, H., Ohmura, Y., Kubo, T., Yoshida, T., & Yoshioka, M. (2018). Assessment of impulsivity in adolescent mice: A new training procedure for a 3-choice serial reaction time task. *Behavioural Brain Research*, *343*, 61–70. https://doi.org/10.1016/j.bbr.2018.01.014

Schindler, C. J., Šlamberová, R., Rimanóczy, A., Hnactzuk, O. C., Riley, M. A., & Vathy, I. (2004). Field-specific changes in hippocampal opioid mRNA, peptides, and receptors due to prenatal morphine exposure in adult male rats. *Neuroscience*, *126*(2), 355–364. https://doi.org/10.1016/j.neuroscience.2004.03.040

Schluger, J. H., Ho, A., Borg, L., Porter, M., Maniar, S., Gunduz, M., Perret, G., King, A., & Kreek, M. J. (1998). Nalmefene causes greater hypothalamic-pituitary-adrenal axis activation than naloxone in normal volunteers: Implications for the treatment of alcoholism. *Alcoholism: Clinical and Experimental Research*, *22*(7), 1430–1436. https://doi.org/10.1111/j.1530-0277.1998.tb03931.x

Schmidt, S. D., Costa, A., Rani, B., Nachtigall, E. G., Passani, M. B., Carta, F., Nocentini, A., De Carvalho Myskiw, J., Furini, C. R. G., Supuran, C. T., Izquierdo, I., Blandina, P., & Provensi, G. (2020). The role of carbonic anhydrases in extinction of contextual fear memory. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(27), 16000–16008. https://doi.org/10.1073/pnas.1910690117

Sclafani, A., Bahrani, M., Zukerman, S., & Ackroff, K. (2010). Stevia and saccharin preferences in rats and mice. *Chemical Senses*, *35*(5), 433–443. https://doi.org/10.1093/chemse/bjq033

Seelke, A. M. H., & Blumberg, M. S. (2005). Thermal and nutritional modulation of sleep in infant rats. *Behavioral Neuroscience*, *119*(2), 603–611. https://doi.org/10.1037/0735-7044.119.2.603

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. In *Progress in Neurobiology* (Vols. 106–107, pp. 1–16). Pergamon. https://doi.org/10.1016/j.pneurobio.2013.04.001

Seney, M. L., Kim, S. M., Glausier, J. R., Hildebrand, M. A., Xue, X., Zong, W., Wang, J., Shelton, M. A., Phan, B. D. N., Srinivasan, C., Pfenning, A. R., Tseng, G. C., Lewis, D. A., Freyberg, Z., & Logan, R. W. (2021). Transcriptional Alterations in Dorsolateral Prefrontal Cortex and Nucleus Accumbens Implicate Neuroinflammation and Synaptic Remodeling in Opioid Use Disorder. *Biological Psychiatry*, *90*(8), 550–562. https://doi.org/10.1016/j.biopsych.2021.06.007

Shaffer, P. M., Rodriguez, C. P., Gaba, A., Byrne, T., Casey, S. C., Harter, J., & Smelson, D. (2021). Engaging vulnerable populations in drug treatment court: Six month outcomes from a co-occurring disorder wraparound intervention. *International Journal of Law and Psychiatry*, *76*. https://doi.org/10.1016/j.ijlp.2021.101700

Shahi, S. K., Freedman, S. N., Dahl, R. A., Karandikar, N. J., & Mangalam, A. K. (2019). Scoring disease in an animal model of multiple sclerosis using a novel infrared-based automated activity-monitoring system. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-55713-7

Shantna, K., Chaudhury, S., Verma, A., & Singh, A. (2009). Comorbid psychiatric disorders in substance dependence patients: A control study. *Industrial Psychiatry Journal*, *18*(2), 84. https://doi.org/10.4103/0972-6748.62265

Shen, Y. L., Chen, S. T., Chan, T. Y., Hung, T. W., Tao, P. L., Liao, R. M., Chan, M. H., & Chen, H. H. (2016). Delayed extinction and stronger drug-primed reinstatement of methamphetamine seeking in rats prenatally exposed to morphine. *Neurobiology of Learning and Memory*, *128*, 56–64. https://doi.org/10.1016/j.nlm.2015.12.002

Siddiqui, A., Haq, S., & Shah, B. H. (1997). Perinatal exposure to morphine disrupts brain norepinephrine, ovarian cyclicity, and sexual receptivity in rats. *Pharmacology Biochemistry and Behavior*, *58*(1), 243–248. https://doi.org/10.1016/S0091-3057(97)00012-9

Simmons, M. L., & Chavkin, C. (1996). Endogenous opioid regulation of hippocampal function. *International Review of Neurobiology*, *39*(39), 145–196. https://doi.org/10.1016/s0074-7742(08)60666-2

Sinclair, D., Purves-Tyson, T. D., Allen, K. M., & Weickert, C. S. (2014). Impacts of stress and sex hormones on dopamine neurotransmission in the adolescent brain. In *Psychopharmacology* (Vol. 231, Issue 8, pp. 1581–1599). Springer Verlag. https://doi.org/10.1007/s00213-013-3415-z

Singh-Taylor, A., Korosi, A., Molet, J., Gunn, B. G., & Baram, T. Z. (2015). Synaptic rewiring of stress-sensitive neurons by early-life experience: A mechanism for resilience? In *Neurobiology of Stress* (Vol. 1, Issue 1, pp. 109–115). Elsevier Inc. https://doi.org/10.1016/j.ynstr.2014.10.007

Sirnes, E., Oltedal, L., Bartsch, H., Eide, G. E., Elgen, I. B., & Aukland, S. M. (2017). Brain morphology in school-aged children with prenatal opioid exposure: A structural MRI study. *Early Human Development*, *106–107*, 33–39. https://doi.org/10.1016/j.earlhumdev.2017.01.009

Siu, A., & Robinson, C. A. (2014). Neonatal abstinence syndrome: essentials for the practitioner. *The Journal of Pediatric Pharmacology and Therapeutics : JPPT : The Official Journal of PPAG*, *19*(3), 147–155. https://doi.org/10.5863/1551-6776-19.3.147

Sjøgren, P., Christrup, L. L., Petersen, M. A., & Højsted, J. (2005). Neuropsychological assessment of chronic non-malignant pain patients treated in a multidisciplinary pain centre. *European Journal of Pain*, *9*(4), 453. https://doi.org/10.1016/j.ejpain.2004.10.005

Skumlien, M., Ibsen, I. O., Kesmodel, U. S., & Nygaard, E. (2020a). Sex Differences in Early Cognitive Development after Prenatal Exposure to Opioids. *Journal of Pediatric Psychology*, *45*(5), 475–485. https://doi.org/10.1093/jpepsy/jsaa008

Skumlien, M., Ibsen, I. O., Kesmodel, U. S., & Nygaard, E. (2020b). Sex Differences in Early Cognitive Development after Prenatal Exposure to Opioids. *Journal of Pediatric Psychology*, *45*(5), 475–485. https://doi.org/10.1093/jpepsy/jsaa008

Šlamberová, R., Bar, N., & Vathy, I. (2003). Long-Term Effects of Prenatal Morphine Exposure on Maternal Behaviors Differ from the Effects of Direct Chronic Morphine Treatment. *Developmental Psychobiology*, *43*(4), 281–289. https://doi.org/10.1002/dev.10141

Šlamberová, R., Hrubá, L., Bernášková, K., Matějovská, I., & Rokyta, R. (2010). Effect of cross-fostering on seizures in adult male offspring of methamphetamine-treated rat mothers. *International Journal of Developmental Neuroscience*, *28*(6), 429–435. https://doi.org/10.1016/j.ijdevneu.2010.06.009

Šlamberová, R., Rimanóczy, Á., Bar, N., Schindler, C. J., & Vathy, I. (2003). Density of μ-opioid receptors in the hippocampus of adult male and female rats is altered by prenatal morphine exposure and gonadal hormone treatment. *Hippocampus*, *13*(4), 461–471. https://doi.org/10.1002/hipo.10076

Šlamberová, R., Rimanóczy, Á., Riley, M. A., & Vathya, I. (2004). Hypothalamo-pituitary-adrenal axis-regulated stress response and negative feedback sensitivity is altered by prenatal morphine exposure in adult female rats. *Neuroendocrinology*, *80*(3), 192–200. https://doi.org/10.1159/000082359

Šlamberová, R., Schindler, C. J., Pometlová, M., Urkuti, C., Purow-Sokol, J. A., & Vathy, I. (2001). Prenatal morphine exposure differentially alters learning and memory in male and female rats. *Physiology and Behavior*, *73*(1–2), 93–103. https://doi.org/10.1016/S0031-9384(01)00469-3

Šlamberová, R., Schindler, C. J., & Vathy, I. (2002). Impact of maternal morphine and saline injections on behavioral responses to a cold water stressor in adult male and female progeny. *Physiology and Behavior*, *75*(5), 723–732. https://doi.org/10.1016/S0031-9384(02)00669-8

Sobor, M., Timár, J., Riba, P., Király, K. P., Gyarmati, S., Al-Khrasani, M., & Fürst, S. (2010). Does the effect of morphine challenge change on maternal behaviour of dams chronically treated with morphine during gestation and further on during lactation? *Pharmacology Biochemistry and Behavior*, *95*(3), 367–374. https://doi.org/10.1016/j.pbb.2010.02.015

Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X. F., Hall, F. S., & Uhl, G. R. (2001). μ Opiate receptor gene dose effects on different morphine actions: Evidence for differential in vivo μ receptor reserve. *Neuropsychopharmacology*, *25*(1), 41–54. https://doi.org/10.1016/S0893-133X(00)00252-9

St. Marie, B., Coleman, L., Vignato, J. A., Arndt, S., & Segre, L. S. (2020). Use and Misuse of Opioid Pain Medications by Pregnant and Nonpregnant Women. *Pain Management Nursing*, *21*(1), 90–93. https://doi.org/10.1016/j.pmn.2019.05.002

Stalnaker, T. A., Takahashi, Y., Roesch, M. R., & Schoenbaum, G. (2009). Neural substrates of cognitive inflexibility after chronic cocaine exposure. In *Neuropharmacology* (Vol. 56, Issue SUPPL. 1, pp. 63–72). Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2008.07.019

Stefano, G. B., Pilonis, N., Ptacek, R., & Kream, R. M. (2017). Reciprocal evolution of opiate science from medical and cultural perspectives. In *Medical Science Monitor* (Vol. 23, pp. 2890–2896). International Scientific Information, Inc. https://doi.org/10.12659/MSM.905167

Stephens, D. N., Duka, T., Crombag, H. S., Cunningham, C. L., Heilig, M., & Crabbe, J. C. (2010). Reward sensitivity: Issues of measurement, and achieving consilience between human and animal phenotypes. In *Addiction Biology* (Vol. 15, Issue 2, pp. 146–168). https://doi.org/10.1111/j.1369-1600.2009.00193.x

Stephens, M. A. C., & Wand, G. (2012). Stress and the HPA axis: Role of glucocorticoids in alcohol dependence. In *Alcohol Research: Current Reviews* (Vol. 34, Issue 4, pp. 468–483). National Institute on Alcohol Abuse and Alcoholism.

Stotts, A. L., Dodrill, C. L., & Kosten, T. R. (n.d.). *Opioid Dependence Treatment: Options In Pharmacotherapy*. https://doi.org/10.1517/14656560903037168

Sugin, L. J. S., Murugesan, A., Bindu, M., & Sunil, K. N. (2020). Roflumilast: A potential drug for the treatment of cognitive impairment? In *Neuroscience Letters* (Vol. 736). Elsevier Ireland Ltd. https://doi.org/10.1016/j.neulet.2020.135281

Sun, R., Mendez, D., & Warner, K. E. (2021). Trends in Nicotine Product Use among US Adolescents, 1999-2020. *JAMA Network Open*, *4*(8), 2118788. https://doi.org/10.1001/jamanetworkopen.2021.18788

Surratt, H. L., Staton, M., Leukefeld, C. G., Oser, C. B., & Webster, J. M. (2018). Patterns of buprenorphine use and risk for re-arrest among highly vulnerable opioid-involved women released from jails in rural Appalachia. *Journal of Addictive Diseases*, *37*(1–2), 1–4. https://doi.org/10.1080/10550887.2018.1531738

Sylvetsky, A. C., Gardner, A. L., Bauman, V., Blau, J. E., Garraffo, H. M., Walter, P. J., & Rother, K. I. (2015). Nonnutritive Sweeteners in Breast Milk. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *78*(16), 1029–1032. https://doi.org/10.1080/15287394.2015.1053646

Taki, K., Kaneko, T., & Mizuno, N. (2000). A group of cortical interneurons expressing μ-opioid receptor-like immunoreactivity: A double immunofluorescence study in the rat cerebral cortex. *Neuroscience*, *98*(2), 221–231. https://doi.org/10.1016/S0306-4522(00)00124-X

Tan, J. W., Duan, T. T., Zhou, Q. X., Ding, Z. Y., Jing, L., Cao, J., Wang, L. P., Mao, R. R., & Xu, L. (2015). Impaired contextual fear extinction and hippocampal synaptic plasticity in adult rats induced by prenatal morphine exposure. *Addiction Biology*, *20*(4), 652–662. https://doi.org/10.1111/adb.12158

Tang, F., Ng, C. M., Bada, H. S., & Leggas, M. (2021). Clinical pharmacology and dosing regimen optimization of neonatal opioid withdrawal syndrome treatments. In *Clinical and Translational Science* (Vol. 14, Issue 4, pp. 1231–1249). John Wiley and Sons Inc. https://doi.org/10.1111/cts.12994

Tarter, R. E., Kirisci, L., Cochran, G., Seybert, A., Reynolds, M., & Vanyukov, M. (2020). Forecasting Opioid Use Disorder at 25 Years of Age in 16-Year-Old Adolescents. *Journal of Pediatrics*, *225*, 207-213.e1. https://doi.org/10.1016/j.jpeds.2020.07.025

Tejeda, H. A., Counotte, D. S., Oh, E., Ramamoorthy, S., Schultz-Kuszak, K. N., Bäckman, C. M., Chefer, V., O'Donnell, P., & Shippenberg, T. S. (2013). Prefrontal

cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. *Neuropsychopharmacology*, *38*(9), 1770–1779. https://doi.org/10.1038/npp.2013.76

Tejeda, H. A., Natividad, L. A., Orfila, J. E., Torres, O. V., & O'Dell, L. E. (2012). Dysregulation of kappa-opioid receptor systems by chronic nicotine modulate the nicotine withdrawal syndrome in an age-dependent manner. *Psychopharmacology*, *224*(2), 289–301. https://doi.org/10.1007/s00213-012-2752-7

Thiele, T. E., & Navarro, M. (2014). "Drinking in the dark" (DID) procedures: A model of binge-like ethanol drinking in non-dependent mice. In *Alcohol* (Vol. 48, Issue 3, pp. 235–241). Elsevier Inc. https://doi.org/10.1016/j.alcohol.2013.08.005

Thomas, A. M., Ostroumov, A., Kimmey, B. A., Taormina, M. B., Holden, W. M., Kim, K., Brown-Mangum, T., & Dani, J. A. (2018). Adolescent Nicotine Exposure Alters GABA A Receptor Signaling in the Ventral Tegmental Area and Increases Adult Ethanol Self-Administration. *Cell Reports*, *23*(1), 68–77. https://doi.org/10.1016/j.celrep.2018.03.030

Thomas, J. H. L., Lui, L., Abell, A., Tieu, W., Somogyi, A. A., Bajic, J. E., & Hutchinson, M. R. (2022). Toll-like receptors change morphine-induced antinociception, tolerance and dependence: Studies using male and female TLR and signalling gene KO mice. *Brain, Behavior, and Immunity*, *102*, 71–85. https://doi.org/10.1016/j.bbi.2022.02.001

Timár, J., Sobor, M., Király, K. P., Gyarmati, S., Riba, P., Al-Khrasani, M., & Fürst, S. (2010). Peri, pre and postnatal morphine exposure: Exposure-induced effects and sex differences in the behavioural consequences in rat offspring. *Behavioural Pharmacology*, *21*(1), 58–68. https://doi.org/10.1097/FBP.0b013e3283359f39

Tobon, A. L., Habecker, E., & Forray, A. (2019a). Opioid Use in Pregnancy. In *Current Psychiatry Reports* (Vol. 21, Issue 12). Springer. https://doi.org/10.1007/s11920-019-1110-4

Tobon, A. L., Habecker, E., & Forray, A. (2019b). Opioid Use in Pregnancy. In *Current Psychiatry Reports* (Vol. 21, Issue 12). Springer. https://doi.org/10.1007/s11920-019-1110-4

Toll, L., Bruchas, M. R., Calo', G., Cox, B. M., & Zaveri, N. T. (2016). Nociceptin/orphanin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. In *Pharmacological Reviews* (Vol. 68, Issue 2, pp. 419–457). American Society for Pharmacology and Experimental Therapy. https://doi.org/10.1124/pr.114.009209

Toorie, A. M., Vassoler, F. M., Qu, F., Slonim, D., Schonhoff, C. M., & Byrnes, E. M. (2022). Intergenerational effects of preconception opioids on glucose homeostasis and hepatic transcription in adult male rats. *Scientific Reports*, *12*(1), 1599. https://doi.org/10.1038/s41598-022-05528-w

Torkaman-Boutorabi, A., Seifi, F., Akbarabadi, A., Toolee, H., Sadat-Shirazi, M.-S., Vousooghi, N., & Zarrindast, M.-R. (2019). *Morphine exposure causes to enhance depression-like behaviour in confront with chronic stress in adult male offspring rat*. *10*(4). https://doi.org/10.32598/bcn.9.10.155

Torres, O. V., Gentil, L. G., Natividad, L. A., Carcoba, L. M., & O'Dell, L. E. (2013). Behavioral, Biochemical, and Molecular Indices of Stress are Enhanced in Female Versus Male Rats Experiencing Nicotine Withdrawal. *Frontiers in Psychiatry*, *4*. https://doi.org/10.3389/fpsyt.2013.00038

Torres, O. V, Pipkin, J. A., Ferree, P., Carcoba, L. M., & O'Dell, L. E. (2014). Nicotine withdrawal increases stress-associated genes in the nucleus accumbens of female rats in a hormone-dependent manner. *Nicotine and Tobacco Research*, *17*(4), 422–430. https://doi.org/10.1093/ntr/ntu278

Torres, O. V., Walker, E. M., Beas, B. S., & O'Dell, L. E. (2014). Female Rats Display Enhanced Rewarding Effects of Ethanol That Are Hormone Dependent. *Alcoholism: Clinical and Experimental Research*, *38*(1), 108–115. https://doi.org/10.1111/acer.12213

Tripathi, S. J., Chakraborty, S., Srikumar, B. N., Raju, T. R., & Shankaranarayana Rao, B. S. (2017). Inactivation of basolateral amygdala prevents chronic immobilization stress-induced memory impairment and associated changes in corticosterone levels. *Neurobiology of Learning and Memory*, *142*, 218–229. https://doi.org/10.1016/j.nlm.2017.05.005

Tschida, K., Michael, V., Takatoh, J., Han, B. X., Zhao, S., Sakurai, K., Mooney, R., & Wang, F. (2019). A Specialized Neural Circuit Gates Social Vocalizations in the Mouse. *Neuron*, *103*(3), 459-472.e4. https://doi.org/10.1016/j.neuron.2019.05.025

Tsukamoto, H., Machida, K., Dynnyk, A., & Mkrtchyan, H. (2009). "Second hit" models of alcoholic liver disease. In *Seminars in Liver Disease* (Vol. 29, Issue 2, pp. 178–187). NIH Public Access. https://doi.org/10.1055/s-0029-1214373

Tucker, L. B., & McCabe, J. T. (2021). Measuring Anxiety-Like Behaviors in Rodent Models of Traumatic Brain Injury. In *Frontiers in Behavioral Neuroscience* (Vol. 15). Frontiers Media S.A. https://doi.org/10.3389/fnbeh.2021.682935

Turano, A., Osborne, B. F., & Schwarz, J. M. (2019). Sexual Differentiation and Sex Differences in Neural Development. In *Current Topics in Behavioral Neurosciences* (Vol. 43, pp. 69–110). Springer. https://doi.org/10.1007/7854_2018_56

Turchan, J., Przewlocka, B., Toth, G., Lasoń, W., Borsodi, A., & Przewlocki, R. (1999). The effect of repeated administration of morphine, cocaine and ethanol on mu and delta opioid receptor density in the nucleus accumbens and striatum of the rat. *Neuroscience*, *91*(3), 971–977. https://doi.org/10.1016/S0306-4522(98)00637-X

Upadhyay, J., Maleki, N., Potter, J., Elman, I., Rudrauf, D., Knudsen, J., Wallin, D., Pendse, G., McDonald, L., Griffin, M., Anderson, J., Nutile, L., Renshaw, P., Weiss, R., Becerra, L., & Borsook, D. (2010). Alterations in brain structure and functional connectivity in prescription opioid-dependent patients. *Brain*, *133*(7), 2098–2114. https://doi.org/10.1093/brain/awq138

Van Cruchten, S., Vrolyk, V., Perron Lepage, M.-F., Baudon, M., Voute, H., Schoofs, S., Haruna, J., Benoit-Biancamano, M.-O., Ruot, B., & Allegaert, K. (2017). Pre- and Postnatal Development of the Eye: A Species Comparison. *Birth Defects Research*, *109*(19), 1540–1567. https://doi.org/10.1002/bdr2.1100

van den Hoogen, N. J., Kwok, C. H. T., & Trang, T. (2021). Identifying the Neurodevelopmental Differences of Opioid Withdrawal. *Cellular and Molecular Neurobiology*, *41*(5), 1145–1155. https://doi.org/10.1007/s10571-020-01035-6

van Hoogdalem, M. W., Wexelblatt, S. L., Akinbi, H. T., Vinks, A. A., & Mizuno, T. (2022). A review of pregnancy-induced changes in opioid pharmacokinetics, placental transfer, and fetal exposure: Towards fetomaternal physiologically-based pharmacokinetic modeling to improve the treatment of neonatal opioid withdrawal syndrome. In *Pharmacology and Therapeutics* (Vol. 234). Elsevier Inc. https://doi.org/10.1016/j.pharmthera.2021.108045

Vathy, I. (1995). Effects of prenatal morphine and cocaine on postnatal behaviors and brain neurotransmitters. In *NIDA research monograph* (Vol. 158, pp. 88–114). NIDA Res Monogr.

Vathy, I., Šlamberová, R., & Liu, X. (2007). Foster mother care but not prenatal morphine exposure enhances cocaine self-administration in young adult male and female rats. *Developmental Psychobiology*, *49*(5), 463–473. https://doi.org/10.1002/dev.20240

Vathy, I., Šlamberová, R., Rimanóczy, Á., Riley, M. A., & Bar, N. (2003). Autoradiographic evidence that prenatal morphine exposure sex-dependently alters μ-opioid receptor densities in brain regions that are involved in the control of drug abuse and other motivated behaviors. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *27*(3), 381–393. https://doi.org/10.1016/S0278-5846(02)00355-X

Velíšek, L., Šlamberová, R., & Vathy, I. (2003). Prenatal morphine exposure suppresses mineralocorticoid receptor-dependent basal synaptic transmission and synaptic plasticity in the lateral perforant path in adult male rats. *Brain Research Bulletin*, *61*(6), 571–576. https://doi.org/10.1016/S0361-9230(03)00194-1

Villalonga-Olives, E., Almansa, J., Shaya, F., & Kawachi, I. (2020). Perceived social capital and binge drinking in older adults: The Health and Retirement Study, US data from 2006–2014. *Drug and Alcohol Dependence*, *214*. https://doi.org/10.1016/j.drugalcdep.2020.108099

Villarreal, D. M., Derrick, B., & Vathy, I. (2008). Prenatal Morphine Exposure Attenuates the Maintenance of Late LTP in Lateral Perforant Path Projections to the Dentate Gyrus and the CA3 Region In Vivo. *J Neurophysiol*, *99*, 1235–1242. https://doi.org/10.1152/jn.00981.2007

Vodo, S., Arcelli, D., Fiorenzani, P., Meriggiola, M. C., Butkevich, I., Di Canio, C., Mikhailenko, V., & Aloisi, A. M. (2013). Gonadal ERα/β, AR and TRPV1 gene expression: Modulation by pain and morphine treatment in male and female rats. *Physiology and Behavior*, *110–111*, 80–86. https://doi.org/10.1016/j.physbeh.2012.12.014

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents. *ILAR Journal*, *55*(2), 310–332. https://doi.org/10.1093/ilar/ilu013

Vousooghi, N., Sadat-Shirazi, M. S., Safavi, P., Zeraati, R., Akbarabadi, A., Makki, S. M., Nazari, S., & Zarrindast, M. R. (2018a). Adult rat morphine exposure changes morphine preference, anxiety, and the brain expression of dopamine receptors in male offspring. *International Journal of Developmental Neuroscience*, *69*, 49–59. https://doi.org/10.1016/j.ijdevneu.2018.06.008

Vousooghi, N., Sadat-Shirazi, M. S., Safavi, P., Zeraati, R., Akbarabadi, A., Makki, S. M., Nazari, S., & Zarrindast, M. R. (2018b). Adult rat morphine exposure changes morphine preference, anxiety, and the brain expression of dopamine receptors in male offspring. *International Journal of Developmental Neuroscience*, *69*, 49–59. https://doi.org/10.1016/j.ijdevneu.2018.06.008

Vuong, C., Van Uum, S. H. M., O'Dell, L. E., Lutfy, K., & Friedman, T. C. (2010). The effects of opioids and opioid analogs on animal and human endocrine systems. In *Endocrine Reviews* (Vol. 31, Issue 1, pp. 98–132). Endocr Rev. https://doi.org/10.1210/er.2009-0009

Wagner, G. C., Jarvis, M. F., Gottesfeld, D. S., Giorlando, M., & Rabii, J. (1986). The effects of prenatal morphine on the responsiveness to morphine and amphetamine. *Pharmacology, Biochemistry and Behavior*, *24*(3), 757–759. https://doi.org/10.1016/0091-3057(86)90586-1

Walters, C. L., Cleck, J. N., Kuo, Y. C., & Blendy, J. A. (2005). μ-opioid receptor and CREB activation are required for nicotine reward. *Neuron*, *46*(6), 933–943. https://doi.org/10.1016/j.neuron.2005.05.005

Wand, G. S., McCaul, M., Yang, X., Reynolds, J., Gotjen, D., Lee, S., & Ali, A. (2002). The mu-opioid receptor gene polymorphism (A118G) alters HPA axis activation induced by opioid receptor blockade. *Neuropsychopharmacology*, *26*(1), 106–114. https://doi.org/10.1016/S0893-133X(01)00294-9

Wang, X., Su, H., Copenhagen, L. D., Vaishnav, S., Pieri, F., Shope, C. Do, Brownell, W. E., De Biasi, M., Paylor, R., & Bradley, A. (2002). Urocortin-Deficient Mice Display Normal Stress-Induced Anxiety Behavior and Autonomic Control but an Impaired Acoustic Startle Response. *Molecular and Cellular Biology*, *22*(18), 6605–6610. https://doi.org/10.1128/mcb.22.18.6605-6610.2002

Wang, Y., Yao, Y., Li, Y., Nie, H., & He, X. (2017). Prenatal morphine exposure during late embryonic stage enhances the rewarding effects of morphine and induces the loss of membrane-bound protein kinase C-α in intermediate medial mesopallium in the chick. *Neuroscience Letters*, *639*, 25–30. https://doi.org/10.1016/j.neulet.2016.12.030

Wang, Y., Yao, Y., Nie, H., & He, X. (2017). Implication of protein kinase C of the left intermediate medial mesopallium in memory impairments induced by early prenatal morphine exposure in one-day old chicks. *European Journal of Pharmacology*, *795*, 94–100. https://doi.org/10.1016/j.ejphar.2016.12.011

Wang, Z., & Storm, D. R. (2011). Maternal Behavior is Impaired in Female Mice Lacking Type 3 Adenylyl Cyclase. *Neuropsychopharmacology*, *36*, 772–781. https://doi.org/10.1038/npp.2010.211

Wasz-Höckert, O., Michelsson, K., & Lind, J. (1985). Twenty-Five Years of Scandinavian Cry Research. In *Infant Crying* (pp. 83–104). Springer US. https://doi.org/10.1007/978-1-4613-2381-5_4

Waszkiewicz, N., Galińska-Skok, B., Nestsiarovich, A., Kułak-Bejda, A., Wilczyńska, K., Simonienko, K., Kwiatkowski, M., & Konarzewska, B. (2018). Neurobiological effects of binge drinking help in its detection and differential diagnosis from alcohol dependence. In *Disease Markers* (Vol. 2018). Hindawi Limited. https://doi.org/10.1155/2018/5623683

Wehbeh, L., & Dobs, A. S. (2020). Opioids and the Hypothalamic-Pituitary-Gonadal (HPG) Axis. In *Journal of Clinical Endocrinology and Metabolism* (Vol. 105, Issue 9, pp. E3105–E3113). Endocrine Society. https://doi.org/10.1210/clinem/dgaa417

Weller, A. E., Crist, R. C., Reiner, B. C., Doyle, G. A., & Berrettini, W. H. (2020). Neonatal Opioid Withdrawal Syndrome (NOWS): A Transgenerational Echo of the Opioid Crisis. *Cold Spring Harbor Perspectives in Medicine*, a039669. https://doi.org/10.1101/cshperspect.a039669

Welle-Strand, G. K., Skurtveit, S., Jones, H. E., Waal, H., Bakstad, B., Bjarkø, L., & Ravndal, E. (2013). Neonatal outcomes following in utero exposure to methadone or buprenorphine: A National Cohort Study of opioid-agonist treatment of Pregnant Women in Norway from 1996 to 2009. *Drug and Alcohol Dependence*, *127*(1–3), 200–206. https://doi.org/10.1016/j.drugalcdep.2012.07.001

Wells, K. (2009). Substance abuse and child maltreatment. In *Pediatric clinics of North America* (Vol. 56, Issue 2, pp. 345–362). Pediatr Clin North Am. https://doi.org/10.1016/j.pcl.2009.01.006

Wetherill, R. R., Franklin, T. R., & Allen, S. S. (2016). Ovarian Hormones, Menstrual Cycle Phase, and Smoking: a Review with Recommendations for Future Studies. In *Current Addiction Reports* (Vol. 3, Issue 1). Springer. https://doi.org/10.1007/s40429-016-0093-z

Wiedenmayer, C. P., Goodwin, G. A., & Barr, G. A. (2000). The effect of periaqueductal gray lesions on responses to age-specific threats in infant rats. *Developmental Brain Research*, *120*(2), 191–198. https://doi.org/10.1016/S0165-3806(00)00009-2

Wilcox, M. V., Carlson, V. C. C., Sherazee, N., Sprow, G. M., Bock, R., Thiele, T. E., Lovinger, D. M., & Alvarez, V. A. (2014). Repeated Binge-like ethanol drinking alters ethanol drinking patterns and depresses striatal GABAergic transmission. *Neuropsychopharmacology*, *39*(3), 579–594. https://doi.org/10.1038/npp.2013.230

Wille-Bille, A., Ferreyra, A., Sciangula, M., Chiner, F., Nizhnikov, M. E., & Pautassi, R. M. (2017). Restraint stress enhances alcohol intake in adolescent female rats but reduces alcohol intake in adolescent male and adult female rats. *Behavioural Brain Research*, *332*, 269–279. https://doi.org/10.1016/j.bbr.2017.06.004

Wilson, N. M., Gurney, M. E., Dietrich, W. D., & Atkins, C. M. (2017). Therapeutic benefits of phosphodiesterase 4B inhibition after traumatic brain injury. *PLoS ONE*, *12*(5). https://doi.org/10.1371/journal.pone.0178013

Winkler, A., Búzás, B., Siems, W. E., Heder, G., & Cox, B. M. (1998). Effect of ethanol drinking on the gene expression of opioid receptors, enkephalinase, and angiotensin-converting enzyme in two inbred mice strains. *Alcoholism: Clinical and Experimental Research*, *22*(6), 1262–1271. https://doi.org/10.1111/j.1530-0277.1998.tb03907.x

Witkowski, G., & Szulczyk, P. (2006). Opioid μ receptor activation inhibits sodium currents in prefrontal cortical neurons via a protein kinase A- and C-dependent mechanism. *Brain Research*, *1094*(1), 92–106. https://doi.org/10.1016/j.brainres.2006.03.119

Wu, L. Y., Chen, J. F., Tao, P. L., & Huang, E. Y. K. (2009). Attenuation by dextromethorphan on the higher liability to morphine-induced reward, caused by prenatal exposure of morphine in rat offspring. *Journal of Biomedical Science*, *16*(1), 106. https://doi.org/10.1186/1423-0127-16-106

Wu, P. L., Yang, Y. N., Suen, J. L., Yang, Y. C. S. H., Yang, C. H., & Yang, S. N. (2018). Long-Lasting Alterations in Gene Expression of Postsynaptic Density 95 and Inotropic Glutamatergic Receptor Subunit in the Mesocorticolimbic System of Rat Offspring Born to Morphine-Addicted Mothers. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/5437092

Xu, J., Azizian, A., Monterosso, J., Domier, C. P., Brody, A. L., London, E. D., & Fong, T. W. (2008). Gender effects on mood and cigarette craving during early abstinence and resumption of smoking. *Nicotine and Tobacco Research*, *10*(11), 1653–1661. https://doi.org/10.1080/14622200802412929

Yang, S. N., Huang, L. T., Wang, C. L., Chen, W. F., Yang, C. H., Lin, S. Z., Lai, M. C., Chen, S. J., & Tao, P. L. (2003a). Prenatal administration of morphine decreases CREBSerine-133 phosphorylation and synaptic plasticity range mediated by glutamatergic transmission in the hippocampal CA1 area of cognitive-deficient rat offspring. *Hippocampus*, *13*(8), 915–921. https://doi.org/10.1002/hipo.10137

Yang, S. N., Huang, L.-T., Wang, C.-L., Chen, W.-F., Yang, C.-H., Lin, S.-Z., Lai, M.-C., Chen, S.-J., & Tao, P.-L. (2003b). Prenatal administration of morphine decreases CREBSerine-133 phosphorylation and synaptic plasticity range mediated by glutamatergic transmission in the hippocampal CA1 area of cognitive-deficient rat offspring. *Hippocampus*, *13*(8), 915–921. https://doi.org/10.1002/hipo.10137

Yang, X., Wang, S., Rice, K. C., Munro, C. A., & Wand, G. S. (2008). Restraint stress and ethanol consumption in two mouse strains. *Alcoholism: Clinical and Experimental Research*, *32*(5), 840–852. https://doi.org/10.1111/j.1530-0277.2008.00632.x

Yen, E., Kaneko-Tarui, T., Ruthazer, R., Harvey-Wilkes, K., Hassaneen, M., & Maron, J. L. (2019). Sex-Dependent Gene Expression in Infants with Neonatal Opioid Withdrawal Syndrome. *Journal of Pediatrics*, *214*, 60-65.e2. https://doi.org/10.1016/j.jpeds.2019.07.032

Yeoh, S. L., Eastwood, J., Wright, I. M., Morton, R., Melhuish, E., Ward, M., & Oei, J. L. (2019). Cognitive and Motor Outcomes of Children with Prenatal Opioid Exposure: A

Systematic Review and Meta-analysis. *JAMA Network Open*, *2*(7). https://doi.org/10.1001/jamanetworkopen.2019.7025

Yilmaz, B., Konar, V., Kutlu, S., Sandal, S., Canpolat, S., Gezen, M. R., & Kelestimur, H. (1999). Influence of chronic morphine exposure on serum LH, FSH, testosterone levels, and body and testicular weights in the developing male rat. *Archives of Andrology*, *43*(3), 189–196. https://doi.org/10.1080/014850199262481

Yohn, C. N., Ashamalla, S. A., Bokka, L., Gergues, M. M., Garino, A., & Samuels, B. A. (2019). Social instability is an effective chronic stress paradigm for both male and female mice. *Neuropharmacology*, *160*, 107780. https://doi.org/10.1016/j.neuropharm.2019.107780

Zanni, G., Robinson-Drummer, P. A., Dougher, A. A., Deutsch, H. M., DeSalle, M. J., Teplitsky, D., Vemulapalli, A., Sullivan, R. M., Eisch, A. J., & Barr, G. A. (2021). Maternal continuous oral oxycodone self-administration alters pup affective/social communication but not spatial learning or sensory-motor function. *Drug and Alcohol Dependence*, *221*, 108628. https://doi.org/10.1016/j.drugalcdep.2021.108628

Zedler, B. K., Mann, A. L., Kim, M. M., Amick, H. R., Joyce, A. R., Murrelle, E. L., & Jones, H. E. (2016a). Buprenorphine compared with methadone to treat pregnant women with opioid use disorder: a systematic review and meta-analysis of safety in the mother, fetus and child. In *Addiction* (Vol. 111, Issue 12, pp. 2115–2128). Blackwell Publishing Ltd. https://doi.org/10.1111/add.13462

Zedler, B. K., Mann, A. L., Kim, M. M., Amick, H. R., Joyce, A. R., Murrelle, E. L., & Jones, H. E. (2016b). Buprenorphine compared with methadone to treat pregnant women with opioid use disorder: a systematic review and meta-analysis of safety in the mother, fetus and child. In *Addiction* (Vol. 111, Issue 12, pp. 2115–2128). Blackwell Publishing Ltd. https://doi.org/10.1111/add.13462

Zeman, M. V., Hiraki, L., & Sellers, E. M. (2002). Gender differences in tobacco smoking: Higher relative exposure to smoke than nicotine in women. *Journal of Women's Health and Gender-Based Medicine*, *11*(2), 147–153. https://doi.org/10.1089/152460902753645281

Zhang, C., Xu, Y., Zhang, H. T., Gurney, M. E., & O'Donnell, J. M. (2017). Comparison of the Pharmacological Profiles of Selective PDE4B and PDE4D Inhibitors in the Central Nervous System. *Scientific Reports*, *7*. https://doi.org/10.1038/srep40115

Zhang, F. fang, Wang, H., Zhou, Y. meng, Yu, H. yang, Zhang, M., Du, X., Wang, D., Zhang, F., Xu, Y., Zhang, J. guo, & Zhang, H. T. (2022). Inhibition of phosphodiesterase-4 in the spinal dorsal horn ameliorates neuropathic pain via cAMP-cytokine-Cx43 signaling in mice. *CNS Neuroscience and Therapeutics*, *28*(5), 749–760. https://doi.org/10.1111/cns.13807

Zhang, G. H., Chen, M. L., Liu, S. S., Zhan, Y. H., Quan, Y., Qin, Y. M., & Deng, S. P. (2011). Effects of mother's dietary exposure to acesulfame-K in pregnancy or lactation on the adult offspring's sweet preference. *Chemical Senses*, *36*(9), 763–770. https://doi.org/10.1093/chemse/bjr050

Zhang, G. H., & Sweitzer, S. M. (2008). Neonatal morphine enhances nociception and decreases analgesia in young rats. *Brain Research*, *1199*, 82–90. https://doi.org/10.1016/j.brainres.2007.12.043

Zhang, H., Chaudhury, D., Nectow, A. R., Friedman, A. K., Zhang, S., Juarez, B., Liu, H., Pfau, M. L., Aleyasin, H., Jiang, C., Crumiller, M., Calipari, E. S., Ku, S. M., Morel, C., Tzavaras, N., Montgomery, S. E., He, M., Salton, S. R., Russo, S. J., … Han, M. H. (2019). α1- and β3-Adrenergic Receptor–Mediated Mesolimbic Homeostatic Plasticity Confers Resilience to Social Stress in Susceptible Mice. *Biological Psychiatry*, *85*(3), 226–236. https://doi.org/10.1016/j.biopsych.2018.08.020

Zhang, H., Kalluri, H. V., Bastian, J. R., Chen, H., Alshabi, A., Caritis, S. N., & Venkataramanan, R. (2018). Gestational changes in buprenorphine exposure: A physiologically-based pharmacokinetic analysis. *British Journal of Clinical Pharmacology*, *84*(9), 2075–2087. https://doi.org/10.1111/bcp.13642

Zhang, J., Yang, Z., & Wu, S. U. (2005). Development of cholinergic amacrine cells is visual activity-dependent in the postnatal mouse retina. *Journal of Comparative Neurology*, *484*(3), 331–343. https://doi.org/10.1002/cne.20470

Zhang, S., Hu, S., Fucito, L. M., Luo, X., Mazure, C. M., Zaborszky, L., & Li, C. S. R. (2017). Resting-State Functional Connectivity of the Basal Nucleus of Meynert in Cigarette Smokers: Dependence Level and Gender Differences. *Nicotine & Tobacco Research : Official Journal of the Society for Research on Nicotine and Tobacco*, *19*(4), 452–459. https://doi.org/10.1093/ntr/ntw209

Zhang, Y., Li, H., Li, Y., Sun, X., Zhu, M., Hanley, G., LeSage, G., & Yin, D. (2011). Essential role of toll-like receptor 2 in morphine-induced microglia activation in mice. *Neuroscience Letters*, *489*(1), 43–47. https://doi.org/10.1016/j.neulet.2010.11.063

Zhu, H., & Barr, G. A. (2004). The role of AMPA and metabotropic glutamate receptors on morphine withdrawal in infant rats. *International Journal of Developmental Neuroscience*, *22*(5–6), 379–395. https://doi.org/10.1016/j.ijdevneu.2004.06.005

Zhu, Y., Hsu, M. S., & Pintar, J. E. (1998). Developmental expression of the μ, κ and δ opioid receptor mRNAs in mouse. *Journal of Neuroscience*, *18*(7), 2538–2549. https://doi.org/10.1523/jneurosci.18-07-02538.1998

Zimmermann, U., Rudin, C., Duò, A., Held, L., & Bucher, H. U. (2020). Treatment of opioid withdrawal in neonates with morphine, phenobarbital, or chlorpromazine: a randomized double-blind trial. *European Journal of Pediatrics*, *179*(1), 141–149. https://doi.org/10.1007/s00431-019-03486-6

Zimmermann, U. S., O'Connor, S., & Ramchandani, V. A. (2011). Modeling alcohol self-administration in the human laboratory. *Current Topics in Behavioral Neurosciences*, *13*, 315–353. https://doi.org/10.1007/7854_2011_149

Zissen, M. H., Zhang, G., McKelvy, A., Propst, J. T., Kendig, J. J., & Sweitzer, S. M. (2007). Tolerance, opioid-induced allodynia and withdrawal associated allodynia in infant and young rats. *Neuroscience*, *144*(1), 247–262. https://doi.org/10.1016/j.neuroscience.2006.08.078