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Perinatal Buprenorphine Effects on Offspring Growth, Opioid Withdrawal, and Brain Morphology in Rats

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by

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ABSTRACT

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Opioid use disorder (OUD) impacts 5.6 million people in the US. Buprenorphine (BUP) is a commonly prescribed opioid medication used to treat OUD, including in pregnant women. However, opioid use during pregnancy is associated with poorer infant outcomes including reduced fetal growth, neurodevelopmental deficits, and neonatal opioid withdrawal syndrome (NOWS). Recent clinical data suggests that providing mothers with a lower dose of BUP may result in fewer negative outcomes in infants. Here, a preclinical rodent model of low-dose perinatal BUP exposure was used to study offspring health outcomes in the neonate, juvenile, and adolescent offspring. Dams were given clinically relevant doses of BUP prior to and throughout gestation, and continuing through weaning to mimic human doses and exposure. Although the lowest BUP dose still elicited signs of NOWS in offspring, there were fewer negative effects on overall brain morphology across the early lifespan than that of the higher BUP dose compared to controls.

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CHAPTER 1. INTRODUCTION

Opioid Use Disorder and Medication-Assisted Treatment During Pregnancy (The Good)

In 2021, nearly 5.6 million people in the U.S. had an opioid use disorder (OUD; SAMHSA 2023). In line with the worsening opioid crisis, opioid use among pregnant women has also increased in severity over the last few decades (Cook 2022; Flores et al. 2023). Currently, the American College of Obstetricians and Gynecologists recommends opioid agonist pharmacotherapy, such as buprenorphine (BUP) or methadone, for pregnant women with OUD to prevent withdrawal symptoms and reduce risk of relapse (American College of Obstetricians and Gynecologists 2017). The semi-synthetic opioid BUP acts as a partial opioid agonist that targets the mu opioid-receptors but also displays opioid antagonism by blocking the kappa opioid-receptors within the brain (Devlin 2022; Flores et al. 2023). The use of BUP to treat OUD in pregnant women has an advantage over the use of methadone in that BUP may be given as an outpatient treatment, does not require daily clinic visits, does not cross the placenta as readily as methadone, and has been reported to reduce negative effects on infants (e.g. withdrawal severity, birth weights, head circumference, length of hospital stay) compared to methadone-treated OUD during pregnancy (Kocherlakota 2019; Szeto 1993).

Medication-Assisted Treatment of OUD During Pregnancy (The Bad)

However, any opioid use during pregnancy, including use of medications for OUD treatment, is generally associated with poorer infant outcomes including reduced fetal growth, neurodevelopmental deficits, and neonatal abstinence syndrome (NAS) compared to healthy non-opioid exposed infants (Whiteman et al. 2014; Patrick et al. 2015; Yazdy et al. 2015; Jansson et al. 2019). Neonatal Opioid Withdrawal Syndrome (NOWS) is a form of NAS that displays itself immediately upon birth and results in a variety of symptoms, including seizures,

irritability, loud vocalization, and hyperthermia (Kraft et al. 2017; Weller et al. 2021). Within a clinical setting, NOWS is typically reported upon birth if signs of withdrawal are evident, however; signs such as seizures may be misinterpreted as common infant sleep jitters, leading to underreporting. Furthermore, some infants who are seemingly healthy might not display withdrawal symptoms until 5-7 days post-birth, long after the mother and child have left the hospital's care and lasting for several weeks in some cases. Post-partum follow ups with mothers and infants are also underreported as many mothers do not attend follow up meetings (Patrick et al. 2020; Weller et al. 2021).

Doses of BUP most often prescribed to pregnant women (i.e., mean dose by time of delivery = 15 mg, range 1-24 mg; Martin et al. 2021) often still result in NOWS following birth (Rana et al. 2023). Furthermore, gestational exposure to BUP has also been associated with long-term behavioral and cognitive consequences (Sundelin and Sarman 2013; Yeoh et al. 2019) although long-term outcomes of gestational BUP exposure are largely understudied in humans. For example, one relatively small clinical study focused on the development of 21 children whose mothers had used BUP during pregnancy up to but not beyond preschool age (Sundelin and Sarman 2013). The children in this study were given neurodevelopmental tests and displayed characteristics similar to that of attention deficit hyperactivity disorders. It is possible, that there may also be even more longer-term consequences of BUP exposure as seen in one study exposure as seen in one study tracking children until the age of 18 that found a negative association between any prenatal opioid exposure, and neurocognitive and physical development until adolescence (Yeoh et al. 2019). Thus, tracking perinatally BUP-exposed children beyond their early childhood stages is an important field of clinical research that will yield better

understanding of the causes and association of prenatal opioid exposure and long-term risks to the children.

In summary, these observations suggest that while the use of BUP to treat OUD in pregnant women may have benefits to the mother, the medication may also consequently have negative effects on infants.

Preclinical Findings of Gestational BUP Exposure

Preclinical models of BUP exposure during gestation can provide translational insight into both short and long-term consequences of opioid exposure on offspring, but there are discrepancies in the literature on BUP route of administration, dosage, and frequency among dams (Farid et al. 2008; Kongstorp et al. 2019, Wallin et al. 2019). For example, Kongstorp and colleagues (2019) utilized osmotic mini pumps implanted subcutaneously in the dam to administer BUP doses continuously, whereas Wallin and colleagues (2019) used daily injections to administer BUP.

Inconsistencies also exist within BUP dosage and timing of BUP administrations among dams. Preclinical studies examined have displayed a wide range of doses from as low as to 0.1 mg/kg and 0.3mg/kg (Wallin et al. 2019; Smith et al. 2023) to as high as 1 mg/kg (Kongstorp et al. 2019; Wallin et al. 2019; Elam et al. 2022; Nieto-Estevez et al. 2022). The amount 1.0mg/kg is commonly chosen as rats metabolize BUP (and other drugs for that matter) faster than humans (Konstorp et al. 2019). However, this range of BUP doses would be equivalent to 24-80 mg in an 80kg human, far exceeding ACOG-recommended doses 2-16 mg/daily in pregnant mothers. There are some preclinical studies that are within the recommended BUP dose for pregnant women, such as Smith and colleagues (2023) that used 0.1 mg/kg in mice (human equivalent of 8mg). Nonetheless, no studies to date look at a minimum clinically relevan low dose (2mg).

Furthermore, within these studies, dosing occurs only at different timepoints during gestation (days 7-28, Wu et al. 2014; days 11-21, Elam et al. 2022; Nieto-Estevez et al. 2022) or throughout the gestation of the offspring up until post-natal days (PND) 14 or weaning at PND 21 (Kongstorp et al. 2019; Wallin et al. 2019; Smith et al. 2023). Combined, these discrepancies between studies may explain the conflicting findings in physical deficits and withdrawal symptoms reported (e.g., Kongstorp et al. 2019 and Smith et al. 2023 find lesser to no negative long-term effects within BUP offspring on brain morphology, offspring characteristics, and offspring behavior, especially when compared to other substances. Studies performed by Elam et al. (2023), Nieto-Estevez et al. (2022), and Wallin et al. (2019) did, however, see significant developmental changes within brain morphology, withdrawal symptoms, and offspring characteristics.

Finally, preclinical studies often do not report comprehensive developmental outcomes of the offspring, particularly past the infant stage. Rather, the studies focus primarily on only one or two developmental stages – juvenile and adolescent, or neonate and juvenile. No study examined appeared to test measures of offspring completely from neonates to adolescents. Unfortunately, the lack of experimental design consistency among BUP offspring studies particularly those beyond the infant stage, thus fails to examine the true long-term effects of the gestational BUP exposure in offspring.

Hypothesis and Design

Recent observations in clinical studies show reducing the dose of BUP reduces NOWS (Macfie et al. 2020; Olsen 2020; Shah et al. 2023). Therefore, studies on the utilization of a clinically relevant low dose BUP (L-BUP) compared to a clinically relevant higher dose of BUP (H-BUP) are important and can provide insight on the effects of BUP intake, specifically

regarding treatment prior to pregnancy and gestational development. Thus, the current preclinical study used a perinatal BUP exposed rat model in which dams were given BUP (0, 0.02, or 0.2 mg/kg) prior to mating, throughout gestation, until three weeks post-birth to mimic human dose and exposure. Offspring of BUP-treated dams were tested as neonates (PND 7), juveniles (PND 22), and adolescents (PND 46-48) for NOWS (Barr et al. 2011; Picut et al. 2015; Wallin et al. 2019). We predicted that lower dose, perinatal BUP would decrease mechanical sensitivity to external stimuli, reduce NOWS scores and mitigate altered brain morphology.

CHAPTER 2. MATERIALS AND METHODS

Animals

Adult Wistar rats (N= 9 dams and 4 sires; Charles River, Raleigh, NC), weighing between 225-300 grams at the beginning of the experiments, were housed in groups of 2-3 per cage in a temperature controlled (22ºC) vivarium on a 12/12-hour light dark cycle (lights on at 7:00) with *ad libitum* access to food and water. The rats were allowed to acclimate to the animal facility for at least 5 days before beginning the experiment. BUP-treated dams and untreated sires were mated for this experiment, resulting in a total offspring of 156. One dam died with 12 offspring in utero from fetal dystocia hindering delivery; 31 additional offspring died due to maternal neglect or consumption (See Table 1). All procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of East Tennessee State University.

Buprenorphine Exposure (Preconception, Gestational, and Postnatal)

Dams (n=4-5/group) were weighed and given either normal saline (0.1 ml/kg) or extended-release buprenorphine (BUP; 0.02 or 0.20 mg/kg, subcutaneous injection; Bup-ER-LAB 0.5 mg/ml, Wedgewood Pharmacy, Swedesboro, NJ) every three days for 2 weeks prior to pairing for mating with a sire. Dams continued to receive respective BUP treatments during the mating period, through gestation, and continued until weaning of the offspring at postnatal day (PND) 21. Doses of BUP were chosen to mimic the lower end of the range and mean of the commonly prescribed therapeutic dosing (range: 2-24 mg, mean:14 mg; Martin et al., 2021). Thus, using a weight-based equivalency for an 80 kg human, 0.02 mg/kg (low dose BUP; L-BUP) and 0.2 mg/kg (high dose BUP; H-BUP) in rodents is approximately equivalent to a 1.6

mg and 16 mg BUP dose, respectively, in pregnant women. Dams were separated from sires when pregnancy was apparent, as observed by weight gain.

Litter Characteristics

Following birth, at PND 6, offspring were counted (live and deceased), sexed, weighed (g), and measured for length (tip of nose to base of tail; cm). Neonatal deaths were noted from PND 1-6. Out of the 13 litters born for this experiment, 3 litters (2 H-BUP and 1 L-BUP) of offspring did not survive to PND 7 (See Table 1). Litters remained with their biological mothers and were weighted and measured for length weekly until PND 21. Mechanical sensitivity, naloxone-precipitated withdrawal testing, and brain collection occurred on PND 7 (neonate, pre-wean), PND 22 (juvenile, within 24-hour of wean), or PND 46-48 (adolescent, 3 weeks post-wean). All offspring were weighed before testing began. Only 2 males and 2 females from each litter were used for any given test to ensure genetic variability within the group. Vehicle-treated dams were transitioned to BUP treatments two weeks after weaning and subsequently bred a second time (n=2 for each L-BUP and H-BUP groups).

Table 1 Mortal and numerical attributes of dams and their offspring.

	Saline	L-BUP	H-BUP
Number of Litters (N= 9 dams)	4	4	5
Dam Death	0	0	1
Offspring Number (N = 156)	41	57	58
Viable Offspring Male (%)	27 (66)	21 (51)	15 (48)
Viable Offspring Female (%)	14 (34)	20 (49)	16 (52)
Offspring Deaths	0	16	27

Mechanical Sensitivity

Using an electronic von Frey instrument (Ugo Basile, Stoelting Co, Wood Dale, IL) juvenile and adolescent offspring underwent automated assessment of threshold to mechanical stimuli (neonate offspring were physically incapable of performing the task). An elevated table,

with the top made of mesh wire, was set up with inverted clear observation boxes containing one rat each for testing. The box was placed over the rat so that the foot pads were accessible through the mesh wire. Rats were acclimated to the boxes on the table 10 minutes prior to the beginning of testing. A wire filament attached to the von Frey device was used to poke the middle of the foot pad of the back hind paw of the rat. The device measured the applied force (g) required for the rat to lift its paw from the filament. Rats were poked twice per hind paw (alternating right, left) and the 4 measurements of applied force were averaged.

Naloxone-Precipitated Withdrawal

Somatic withdrawal signs of NOWS were measured immediately following mechanical sensitivity testing. Naloxone-precipitated withdrawal was scored as previously (Barr et al. 2011; Vendruscolo et al. 2011; Wallin et al. 2019), with minor modifications for each developmental stage (neonate, juvenile, and adolescent). Offspring were acclimated to the testing box for 10 minutes and were then given naloxone hydrochloride (1 mg/kg, subcutaneously). After 5 minutes, rats were placed in a clear observation box and observed by two experienced, independent observers for 10 minutes. Sessions were also video recorded for accuracy. Signs of opioid withdrawal included: escape attempts (jumps or wall climbs), wet dog shakes, paw/body/limb tremors, stretching, mastication, defecation/diarrhea, teeth chattering, swallowing movements, salivation, ptosis, genital grooming, abdominal spasms, hyperirritability/vocalization upon touch, abnormal posture, instability/body roll, face grooming, and locomotion/forepaw treading. These signs were specified for the developmental stage examined as seen in Table 2 (for complete withdrawal score sheets see Appendices A-C). Each naloxone-precipitated withdrawal score was calculated as the average sum of observed signs determined by the observers.

Table 2 Opioid withdrawal signs observed at each developmental stage.

Signs of Opioid Withdrawal	**Neonate (PND 7)**	**Juvenile (PND 22)**	**Adolescent (PND 46-48)**
Escape Attempts	# Wall Climbs	# Wall Climbs	# Jump Attempts
Paw/Body/Limb Tremors	+	+	+
Wet Dog Shakes	-	+	+
Stretching	+	-	-
Mastication	+	+	-
Teeth Chattering	-	-	+
Swallowing Movements	-	-	+
Salivation	-	-	+
Ptosis	-	+	+
Face Grooming	+	+	-
Genital Grooming	-	-	+
Abdominal Spasms	-	-	+
Defecation/Diarrhea	+	+	+
Hyperirritability/ Vocalization	+	+	+
Abnormal Posture	+	+	+
Instability/Body Roll	+	-	-
Locomotion/Forepaw Treading	+	+	-

Brain Morphology

Immediately following naloxone-precipitated withdrawal testing, offspring were deeply anesthetized with 5% isoflurane and perfused transcardially with 4% formaldehyde. The brains were removed and placed in fixative prior to conducting whole brain measures. Subsequently, brains were frozen, coronally sectioned (40 μm) on a cryostat (Leica CM 3050 S) and stained with neutral red dye to assess specific brain regions of interest.

Whole Brain Measurements. Prior to sectioning, brains were measured for weight (g), cortical length (cm), and midbrain and cerebral width (cm; Figure 3B). Measures were obtained using ImageJ (v.1.53) from images obtained with an iPhone (13 Pro Max). Cortical length was averaged across the two hemispheres. Midbrain and cerebellar width measurements were taken from a horizontal line across the widest extent of each region.

Brain Regions of Interest. The brain regions analyzed from stained coronal sections were the frontal cortex (FC), prelimbic cortex (PLC), somatosensory cortex (SC), corpus callosum (CC), basal ganglia (BG), hippocampus (CA3), central amygdala (CEA) and basolateral amygdala (BLA). Images of brain regions of interest were obtained from AmScope Light Microscope equipped with AmScopeAmLite software and a 20MP C-mount microscope camera (MU2003-BI-RU1, AmScope, Irvine, CA, USA) and analyzed with ImageJ v.1.53. All regions of interest (ROI; examples, see Figure 4) were measured in micrometers. For the FC regions, the left and right FC were measured as follows: when first observed in the brain tissue, the highest incline of the forceps minor of the corpus callosum was determined, and the line was drawn from this incline to the dorsal edge of the brain tissue. The PLC regions were measured horizontally from just below the beginning curvature of the forceps minor of the corpus callosum (fmi) medially to the midline. For the SC regions, a line was drawn from the caudate putamen to the lateral edge of the brain tissue. For the CC, the widest extent of the CC was measured from the azygous artery ventrally to the genu of the corpus callosum to the bottom. For the BG, the area of the caudate putamen was measured, tracing along the ependyma layer. For the CA3, the CA3 layer at its widest point was measured horizontally. For the BLA, the area between the external capsule medially to the CeA was measured. For the CeA, a spherical area was measured between BLA and the internal capsule. All data for ROIs were analyzed from one hemisphere and within treatment type (SAL, L-BUP, and H-BUP) at each developmental stage.

Data Analysis

Data was analyzed using GraphPad Prism 10. All data, with exception of number of offspring, were analyzed using a between-subjects two-way analysis of variance (ANOVA), with the factors of BUP dose (three levels: SAL, L-BUP, and H-BUP) and age (three levels: NEO,

JUV, and ADOL) or time (six levels: number of weeks 1-6). The number of offspring (pups/litter and deceased) were analyzed using a one-way ANOVA for BUP dose. Naloxone-precipitated withdrawal scores were additionally analyzed using a one-way ANOVA for BUP comparisons at each developmental stage due to the difference in scales used for each. When appropriate, *post hoc* comparisons were performed using Tukey Honest Significant Difference multiple-comparison test. P values < 0.05 were determined to be statistically significant. Sex effects were not analyzed due to small sample sizes.

CHAPTER 3. RESULTS

Maternal and Offspring Growth Outcomes

Both dams and offspring physical growth was tracked during the experiment and analyzed for an effect of dose. Weights for both dams and offspring were tracked, and body length was tracked for offspring only.

Dam Body Weight Percentage. The maternal weights were tracked for six weeks prior to delivery and expressed as a percent increase from start of the experiment (6 weeks pre-delivery). There was main effect of BUP dose on maternal weight gain, with a significant decrease in percentage of weight gain in the H-BUP dams compared to the SAL treated controls (Figure 1A; Dose: $F_{(2,60)} = 5.53$, $p < 0.01$; Time: $F_{(5,60)} = 30.64$, $p < 0.01$; Dose x Time: $F_{(10,60)} = 0.66$, $p = 0.76$).

Number of Offspring. The number of pups per litter and deceased pups before PND 6 were also tracked for each BUP treatment group (Figure 1B). There was no significant effect of BUP dose on either pups/litter ($F_{(2,10)} = 2.17$, $p = 0.16$) or deceased pups ($F_{(2,10)} = 0.81$, $p = 0.47$).

Offspring Body Weight and Length. Overall offspring body weights and lengths across all treatment groups increased post-birth as offspring aged, but there was no main effect of BUP dose on offspring weights (Figure 1C; Weight: Dose: $F_{(2, 412)} = 0.61$, $p = 0.55$; Time: $F_{(5,412)} = 1630$, $p < 0.05$; Dose x Time $F_{(10, 412)} = 0.40$, $p = 0.90$). There was a specific interaction of BUP dose and time that indicated an increase in offspring body length in the H-BUP group at 3 and 4 weeks post-birth and in the L-BUP at 4 weeks post birth compared to SAL controls (Length: Dose: $F_{(2, 372)} = 0.86$, $p = 0.42$; Time: $F_{(5, 372)} = 869$, $p < 0.01$; Dose x Time $F_{(10, 372)} = 2.65$, $p < 0.01$). However, there were no differences between the groups at 1, 2, 5, or 6 weeks following birth.

Precipitated Withdrawal and Mechanical Sensitivity

Naloxone Precipitated Withdrawal. Naloxone-precipitated withdrawal scores were recorded for each developmental age using the appropriate withdrawal score sheets (see Appendices A-C). Omnibus comparisons showed a significant effect of BUP dose on withdrawal score and both L-BUP and H-BUP scores were significantly increased compared to SAL controls (Figure 2A; Dose: $F_{(2,72)} = 7.54$, $p < 0.01$; Age: $F_{(2,72)} = 26.64$, $p < 0.01$; Dose x Time $F_{(4,72)} = 0.37$, $p = 0.83$). Further analysis of each development stage due to use of age-appropriate withdrawal scales showed a significant effect of BUP dose in neonates ($F_{(2,18)} = 4.85$, $p < 0.05$), but not the juveniles ($F_{(2,31)} = 2.06$, $p = 0.15$) or adolescents ($F_{(2,23)} = 2.97$, $p = 0.07$), although adolescents were nearing significance.

Mechanical Sensitivity. Electronic von Frey testing was conducted on juvenile and adolescent offspring only. There was a significant effect of BUP dose on mechanical sensitivity showing a significant increase in force required for paw withdrawal in the H-BUP, but not L-BUP group, compared to SAL controls (Figure 2B; Dose: $F_{(2, 54)} = 4.12$, $p < 0.05$; Age: $F_{(1, 54)} = 225.9$, $p < 0.01$; Dose x Age: $F_{(2,54)} = 0.14$, $p = 0.87$).

Brain Morphology

Brain Weights. There was a significant main effect of BUP dose across the developmental time points on brain weights with H-BUP offspring showing a decrease in brain weight compared to SAL controls (Figure 3A; Dose: $F_{(2,78)} = 3.324$ $p < 0.05$; Age: $F_{(2,78)} = 895.8$, $p < 0.01$; Dose x Age: $F_{(4, 78)} = 0.34$, $p = 0.85$).

Cortical Lengths. There was no significant main effect of BUP dose across the developmental time points on cortical length (Figure 3C; Dose: $F_{(2, 78)} = 0.52$, $p = 0.59$, Age: $F_{(2,78)} = 452.3$, $p < 0.01$; $F_{(4,78)} = 0.93$, $p = 0.45$).

Midbrain and Cerebellar Widths. There was a significant main effect of BUP dose across the developmental time points on both midbrain and cerebellar widths (Figure 3D and E, respectively) with H-BUP offspring showing a decrease in midbrain and cerebellar widths compared to SAL controls (Midbrain: Dose: $F_{(2,77)} = 8.99$; $p < 0.01$; Age, $F_{(2,77)} = 190.6$, $p<0.01$; Dose x Ag: $F_{(4,77)} = 1.64$, $p = 0.17$; Cerebellar: Dose: $F_{(2,72)} = 16.08$, $p < 0.01$; Age $F_{(2,72)} = 279.1$, $p < 0.01$; Dose x Age: $F_{(4,72)} = 1.12$, $p = 0.35$). The L-BUP offspring also showed a significant increase in cerebellar widths compared to H-BUP and SAL controls.

ROIs

Frontal Cortex (FC). There was a main interaction between age and BUP dose (Figure 4D; Age: $F_{(2,61)} = 33.88$, $p < 0.01$; Dose: $F_{(2,61)} = 1.79$, $p = 0.18$; Dose x Age: $F_{(4,61)} = 5.14$, $p < 0.01$), with a significant effect of H-BUP in neonates compared to SAL and L-BUP.

Prelimbic Cortex (PLC). There was a significant main interaction between age and BUP dose with significant difference between L-BUP and both SAL and H-BUP (Figure 4E; Age: $F_{(2, 67)} = 204.9$, $p < 0.01$; Dose: $F_{(2,67)} = 2.99$, $p = 0.06$, Dose x Age: $F_{(4,67)} = 4.04$, $p < 0.01$).

Somatosensory Cortex (SC). There was a significant main effect of age and BUP dose across developmental stages with H-BUP offspring showing a decrease in SC size compared to SAL controls (Figure 4F; Age: $F_{(2,73)}= 87.07$, $p < 0.01$; Dose: $F_{(2,73)} = 3.21$, $p < 0.05$; Dose x Age: $F_{(4, 73)} = 1.20$, $p = 0.32$).

Corpus Callosum (CC). There was no significant main effect of BUP dose across the developmental time points on the depth of the CC (Figure 4J; Age: $F_{(2,68)} = 18.23$, $p < 0.01$; Dose: $F_{(2, 68)} = 2.15$, $p = 0.13$; Dose x Age: $F_{(4, 68)} = 0.48$, $p = 0.75$.

Basal Ganglia (BG). There was no significant main effect of BUP dose across the developmental time points on the size of the BG (Figure 4K; Age: $F_{(2, 69)} = 0.77$, $p = 0.47$; Dose: $F_{(2, 69)} = 136.8$, $p < 0.01$; Dose x Age: $F_{(4, 69)} = 0.73$, $p = 0.58$).

Hippocampus (CA3). There was a significant main effect of BUP dose across developmental stages for a decrease in CA3 size in H-BUP compared to SAL controls (Figure 4L; Age: $F_{(2, 75)} = 5.68$) $p < 0.01$; Dose: $F_{(2,75)} = 4.24$, $p < 0.05$; Dose x Age: $F_{(4, 75)} = 0.43$, $p = 0.43$).

Basolateral Amygdala (BLA). There was no significant main effect of BUP dose across the developmental time points on the size of the BLA (Figure N; Age: $F_{(2, 59)} = 12.62$, $p < 0.01$; Dose: $F_{(2, 59)} = 1.92$, $p = 0.16$; Dose x Age: $F_{(4, 59)} = 1.14$, $p = 0.35$).

Central Amygdala (CEA). There was no significant main effect of BUP dose across the developmental time points on the size of the CEA (Figure 4O; Age: $F_{(2, 58)} = 12.90$, $p < 0.01$; Dose: $F_{(2, 58)} = 2.83$, $p = 0.07$; Dose x Age: $F_{(2, 58)} = 0.79$, $p = 0.54$).

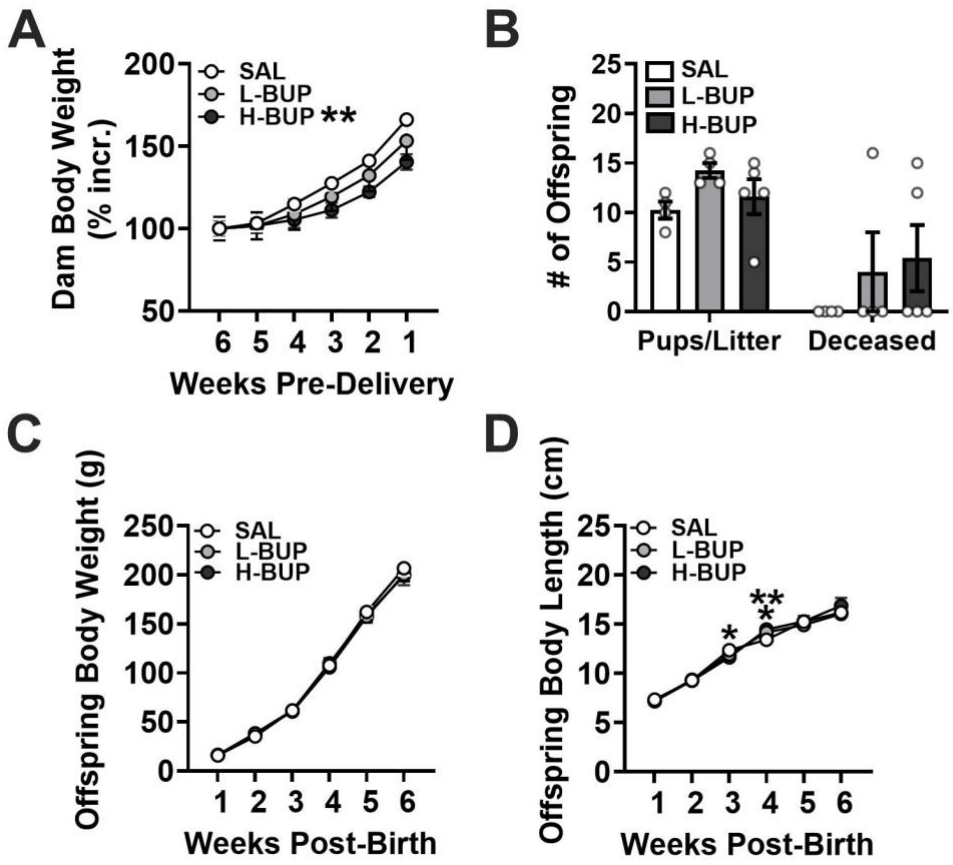

Fig 1. Effects of the BUP on dam weights during pregnancy and offspring growth.

Panel A – Symbols represent the mean (± SEM) body weights expressed as percent increase from 6 weeks for each of 6 weeks pre-delivery of the litters for saline (SAL; white), low dose buprenorphine (L-BUP; light gray) and high dose buprenorphine (H-BUP; dark gray) treated dams. H-BUP treated dams showed significantly less body weight gain prior to delivery. **Panel B –** Bars represent the mean (± SEM) number of pups/litters (left) and pups found deceased (right) from SAL (white), L-BUP (light gray), and H-BUP (dark gray) treated dams. There was no significant difference between the BUP groups. **Panels C and D –** Symbols represent the mean (± SEM) offspring body weights (C) and lengths (D) for each of 6 weeks post-birth for SAL (white), L-BUP (light gray), and H-BUP (dark gray) exposed litters. There was no main effect of BUP dose on offspring body weights; however, offspring body lengths displayed increase in H-BUP body length, observed for weeks 3 and 4, and an increase in L-BUP body length, observed for week 4, both compared to SAL controls. *p, **p < 0.05, 0.01 versus SAL controls.

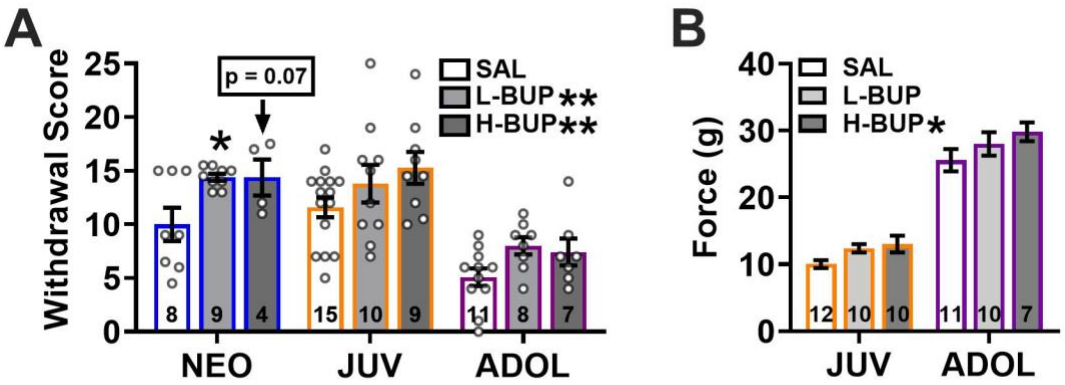

Fig 2. Effects of BUP on withdrawal scores and mechanical sensitivity across developmental stage. **Panel A -** Bars represent the mean (± SEM; n in bars) naloxone-precipitated withdrawal scores across developmental stage for saline (SAL; white), low dose buprenorphine (L-BUP; light gray), and high dose buprenorphine (H-BUP, dark gray) exposed offspring. Both L-BUP and H-BUP dose significantly increased withdrawal scores compared to SAL. In neonates, specifically, there was significant increase in L-BUP withdrawal scores, and H-BUP was also nearing significance ($p = 0.07$). **Panel B –** Bars represent the mean (± SEM; n in bars) mechanical sensitivity measured in grams of force across juvenile and adolescent developmental stages (neonates were physiologically exempt) for SAL (white), L-BUP (light gray), and H-BUP (dark gray) exposed offspring. There was a significant increase in the amount of force (g) needed to withdraw the hind paw in H-BUP, but not L-BUP, compared to SAL. *p, **$p < 0.05$, 0.01 versus SAL controls. Adolescent, ADOL; juvenile, JUV; neonate, NEO.

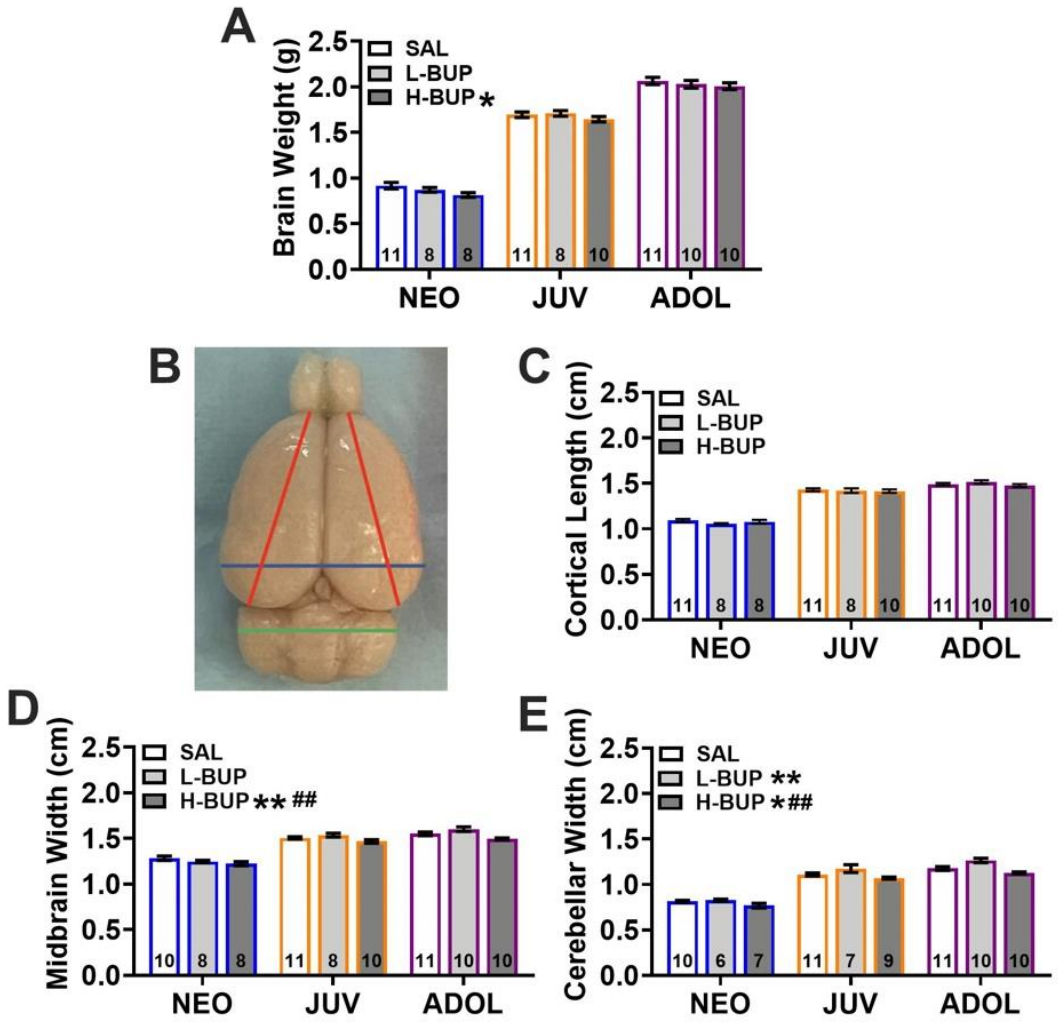

Fig 3. Effects of BUP on overall brain size across offspring developmental stages.
Bars represent the mean (± SEM; n in bars) offspring brain weights (g; **Panel A**), cortical lengths (cm; **Panel C**), midbrain widths (cm; **Panel D**), and cerebellar widths (cm; **Panel E**) across developmental stages for saline (SAL; white), low dose buprenorphine (L-BUP; light gray), and high dose buprenorphine (H-BUP; dark gray). **Panel B –** Representative image of whole brain and measurement line vectors used for cortical length (red line), midbrain width (blue), and cerebellar width (green). There was a significant decrease in offspring brain weights (**A**) in H-BUP, but not to L-BUP, offspring compared to SAL. There was no significant effect of BUP on cortical lengths (**C**). H-BUP exposed offspring had significantly reduced midbrain (**D**) and cerebellar (**E**) widths, and L-BUP exposed offspring also had significantly reduced cerebellar widths (**E**) compared to SAL controls. *p, **p < 0.05, 0.01 versus SAL controls; $^{\#\#}$p < 0.01 versus L-BUP. Adolescent, ADOL; juvenile, JUV; neonate, NEO.

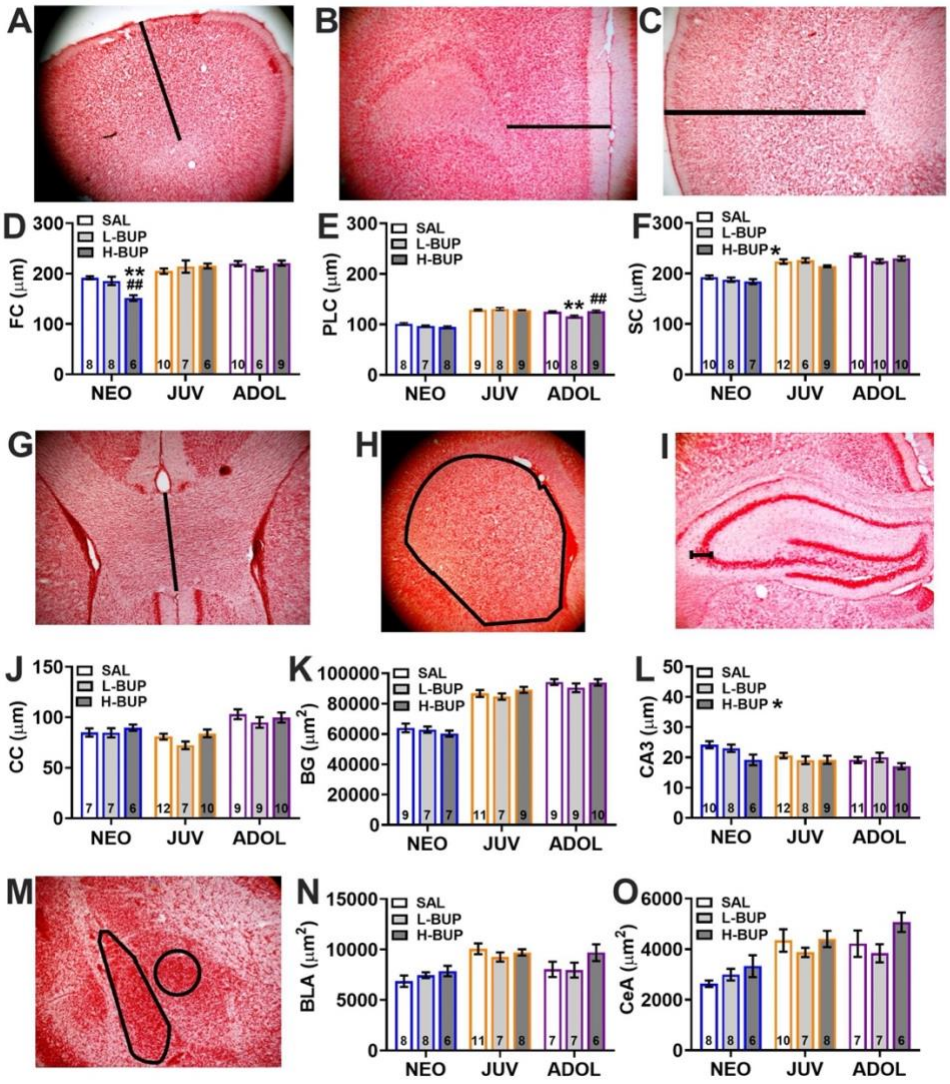

Fig 4. Effects of BUP exposure on specific brain region size across developmental stages. **Panels A-C, G-I, and M –** representative coronal sections of each ROI, outline and line vectors indicated area of measure. Bars represent the mean (± SEM; n in bars) lengths (µm; FC, **Panel D**; PLC, **Panel E**; SC, **Panel F**; CC, **Panel J**; CA3, **Panel L**) and areas (µm^2; BG, **Panel K**; BLA, **Panel N**; CEA, **Panel O**) across developmental stages for saline (SAL, white), low dose buprenorphine (L-BUP, light gray), and high dose buprenorphine (H-BUP, dark gray) exposed offspring. There was a significant decrease in FC thickness within H-BUP neonates compared to L-BUP offspring and SAL controls. There was a significant decrease in PLC thickness within L-BUP neonates compared to SAL controls. There was an overall decrease in SC and CA3 in H-BUP, but not L-BUP, offspring compared to SAL controls across the developmental stages. There were no significant effects of BUP on CC, BG, BLA, or CEA. *p, **p < 0.05, 0.01 versus SAL controls; $^{\#\#}$p < 0.05, 0.01 versus L-BUP. Adolescent, ADOL; basal ganglia, BG; basolateral amygdala, BLA; central amygdala, CEA; corpus callosum, CC; frontal cortex, FC; hippocampus, CA3; juvenile, JUV; neonate, NEO; prelimbic cortex, PLC; somatosensory cortex, SC.

CHAPTER 4. DISCUSSION

The current studies tested a commonly used medication, BUP, for the treatment of OUD in pregnant women in a rodent model of gestational BUP exposure to determine whether a low dose of BUP (i.e., L-BUP) would benefit health outcomes in offspring compared to a higher, yet clinically moderate dose of BUP (i.e., H-BUP). A significant decrease in overall percentage of weight gain was observed among H-BUP dams compared to the control dams. While both L-BUP and H-BUP elicited a significant increase in precipitated withdrawal scores in offspring compared to control offspring, only H-BUP offspring displayed increased mechanical sensitivity during the juvenile and adolescent developmental periods. Furthermore, overall brain weights and widths were significantly reduced only in H-BUP offspring compared to the control offspring across the life span. Additional significant morphological changes occurred in H-BUP offspring in the frontal cortex, somatosensory cortex, and the hippocampus. In short, offspring exposed to H-BUP during gestation through weaning appeared to have more negative health outcomes than L-BUP exposed offspring when compared to saline-exposed controls, suggesting a lower dose of BUP may be recommended for treatment of OUD during pregnancy.

Maternal and Offspring Growth Outcomes

Maternal growth during pregnancy and offspring growth following birth were examined within the experiment. In humans, maternal weight gain tends to impact the overall size of child. If a mother does not gain the appropriate weight during pregnancy, the child runs the risk of being undeveloped in size and unable to reach developmental markers appropriately, such as crawling, walking, and speaking. There is also the risk of a weakened immune system. Inversely, if a mother obtains higher than the recommended weight gain, the child can run the risk of childhood obesity (CDC 2022). Within our experiment, dam body weight gain was significantly

lower among H-BUP-treated dams compared to the saline-treated dams, while L-BUP-treated dams did not gain significantly less weight than controls. Reduced weight gain in H-BUP-treated dams was not due to either smaller overall litter size or offspring body weights (Figure 1, B and C). However, it is possible that dams receiving the higher dose of BUP had reduced food absorption and/or water consumption during their pregnancy resulting from opioid-induced gastric distress, as seen previously in non-pregnant female rats (Liles and Flecknell 1992; Jablonski et al. 2001; Jessen et al. 2006). Maternal health and nutrition while receiving BUP during pregnancy is an important topic for future studies as clinical studies have shown gestational BUP exposure is positively associated with lower birth weights and lengths in infants (Jansson et al. 2017).

Naloxone-Precipitated Withdrawal and Mechanical Sensitivity

Overall naloxone-precipitated withdrawal scores increased in both L-BUP and H-BUP offspring compared to saline-treated controls, suggesting that even the lower clinically relevant dose elicits NOWS in rodents. However, human data has shown a reduction in the likelihood of NOWS when mothers are treated with a lower clinically relevant dose of BUP (Macfie et al. 2020; Olsen 2020; Shah et al. 2023). This may be a species difference or possibly dependent on the length of opioid exposure in mothers prior to conception.

It was hypothesized that offspring exposed to H-BUP would display increased mechanical sensitivity compared to L-BUP or saline exposed control offspring, particularly as withdrawal-induced hyperalgesia is a consequence of chronic opioid use (Wala and Holtman 2011; Higgins et al. 2019). Although mechanical sensitivity was not possible to measure in the neonates due to their inability to retract their hind paw, H-BUP offspring surprisingly displayed a significant decrease in sensitivity (i.e., greater force required for paw withdrawal) compared to

control offspring. This blunted mechanical sensitivity may be attributed to the higher dose of BUP remaining in the H-BUP offspring's system longer than that of L-BUP exposed offspring, resulting in antinociception in H-BUP offspring (Wala and Holtman 2011). Future studies should consider performing naloxone-precipitated withdrawal prior to nociception testing to determine whether a state of withdrawal may increase mechanical sensitivity in BUP exposed offspring.

Brain Morphology

One goal of current studies was to determine whether there are longer term changes to brain morphology in offspring exposed to gestational BUP that could ultimately lead to functional or developmental deficits later in life. A reduction in overall brain weight and midbrain width was observed in H-BUP, but not L-BUP, offspring compared to saline-treated control offspring. However, the cerebellar widths were reduced in both L-BUP and H-BUP offspring compared to the control group, whereas there was no effect of BUP on cortical length. It is not clear as to why the weight and widths displayed reductions whilst the cortical lengths had no BUP dose effect, although this may be due to underpowered group sizes for all measures.

There were also several specific brain regions that showed reductions, particularly in the H-BUP offspring. The FC thickness was decreased in H-BUP offspring compared to both L-BUP and control offspring in neonates, but growth appeared to recover by juvenile and adolescent stages. Although the main effect of dose on PLC thickness was nearing significance ($p=0.06$), there was a significant interaction effect such that L-BUP offspring had decreased PLC depth compared to both H-BUP and SAL offspring during the adolescent time point. There was also a significant overall decrease in SC in H-BUP offspring compared to SAL across all time points. Combined, these observations suggest that BUP has a negative effect on cortical thickness that

could potentially impair higher-order cognitive processing and may translate to cognitive and sensory deficits later in life.

Within the hippocampal CA3, H-BUP offspring displayed significant reduction in thickness compared to control, but not L-BUP, offspring. This reduced hippocampal CA3 might indicate a negative impact to the overall memory and learning processes of BUP offspring and is an important topic for a future study. The CC, BG, CEA, and BLA did not display any significant changes in BUP-treated offspring within this experiment. However, the observed changes to overall and region-specific brain size may ultimately translate to changes in cognition and behavior across the lifespan of the offspring.

Limitations

Due to the unpredictable nature of breeding, some data categories remain lackluster. Continuance of this study will produce a much-needed larger sample size of offspring to subsequently increase power of the statistical analyses. Furthermore, larger sample sizes would allow for investigation into specific sex differences for each measure. As is, there were too few of each sex to adequately assess sex differences in these data. However, the number of offspring tested in this study provides preliminary research that suggests a lower dose of BUP elicits fewer negative outcomes compared to the higher dose of BUP.

Another limitation is that this study is lacking maternal caregiving measures in the dams themselves. However, given there was no difference in offspring overall wellbeing as measured by length and weight over the lifespan, we do not anticipate a maternal caretaking deficit with these very moderate doses of BUP. Furthermore, another preclinical study showed no effect of gestational exposure to BUP on maternal caregiving when examined 5 days following birth and similarly also showed no effect of BUP on offspring growth (Kongstorp et al. Jul. 2020).

Nevertheless, future studies could control for this confound by cross-fostering BUP offspring with saline-treated dams.

Regarding NOWS symptom scoring, naloxone was used to precipitate withdrawal to acutely and timely identify withdrawal symptoms. Here slightly different scoring categories were used for each developmental point (see Appendices A-C), largely depending on the physical capabilities of each developmental stage. A more consistent score sheet that can be used across timepoints for age comparisons may be useful. Additionally, it is also possible to observe withdrawal symptoms during spontaneous withdrawal, which has parallelism to commonly used clinical procedures for NAS/NOWS testing in neonates. Future studies may also consider spontaneous withdrawal observations in this rodent model of gestational BUP exposure.

Finally, in humans, the rationale for opioid agonist therapy for treatment of OUD during pregnancy is to help mothers reach a full-term pregnancy by reducing relapse risk and craving for nonmedical opioids while increasing adherence to prenatal care programs. However, by lowering the dose of BUP, as suggested here, there may in turn be an increased risk of craving leading to BUP or other opioid misuse. Thus, tracking relapse rats in mothers in both preclinical and clinical studies is an important consideration for future studies in that placing mothers at risk will ultimately put infants at risk as well. Furthermore, to better parallel the clinical condition, forthcoming preclinical studies should include dams previously dependent on opioids prior to transition to perinatal BUP treatments. These recommended changes to study design will help researchers better assess the risk of gestational low dose BUP in both mother and their offspring.

CHAPTER 5. CONCLUSIONS

This experiment's findings suggests that there are negative effects of the modestly higher clinical dose of BUP upon mechanical sensitivity, precipitated withdrawal, brain morphology, and specific brain regions of interest; however, the lower clinical dose of BUP still elicited precipitated withdrawal symptoms, although overall there were fewer negative outcomes in offspring exposed to the lowest dose of BUP. This suggests that providing a lower, yet clinically relevant, dose of BUP may provide a means to decrease short- and long-term effects in the children born of pregnant mothers suffering from OUD.

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APPENDICES

Appendix A: Naloxone-Precipitated Withdrawal Score Sheet for Neonates (PND 7)

NEONATES (PN0-7):: Somatic Signs of Opioid Withdrawal 12/19/22

For precipitated withdrawal: administer 1mg/kg Naloxone, s.c., wait 5 mins and place in small (mouse size) clear box; observed signs of withdrawal for 10 min

Rat ID					
Date					
Observer					

Withdrawal Sign		**Points**	**Points**	**Points**	**Points**	**Points**
Graded signs:						
Paw/limb/body Tremors	1-2	2	2	2	2	2
(shaking movements)	3+	4	4	4	4	4
Stretching	1-2	2	2	2	2	2
(Elongation of extremities)	3+	4	4	4	4	4
Mastication	1-3	2	2	2	2	2
(mouth movements/open,close)	4+	4	4	4	4	4
Wall climbs	1-4	2	2	2	2	2
(two front paws touch wall)	5-9	3	3	3	3	3
	10+	4	4	4	4	4
Instability/Body Roll	1-4	2	2	2	2	2
(loss of righting, 1/2 roll)	5-9	3	3	3	3	3
	10+	4	4	4	4	4
Checked signs*:						
** use severity scale: 1= present/mild, 2 = moderate, 3 = intense*						
Face grooming (moving paws across face)		3	3	3	3	3
Abnormal Posture		3	3	3	3	3
Irritability/vocalization (spontaneous or touch)		3	3	3	3	3
Locomotion/Forepaw treading*		1 2 3	1 2 3	1 2 3	1 2 3	1 2 3

Appendix B: Naloxone-Precipitated Withdrawal Score Sheet for Juveniles (PND 22)

JUVENILE (PN21-32):: Somatic Signs of Opioid Withdrawal 12/19/22

For precipitated withdrawal: administer 1mg/kg Naloxone, s.c., wait 5 mins and place in small (mouse size) clear box; observed signs of withdrawal for 10 min

Rat ID					
Date					
Observer					

Withdrawal Sign		**Points**	**Points**	**Points**	**Points**	**Points**
Graded signs:						
Wet dog shakes	1-2	2	2	2	2	2
(sudden movement head/neck/body)	3+	4	4	4	4	4
Paw/limb/body Tremors	1-2	2	2	2	2	2
(shaking movements)	3+	4	4	4	4	4
Mastication	1-3	2	2	2	2	2
(mouth movements/open,close)	4+	4	4	4	4	4
Wall climbs	1-4	2	2	2	2	2
(two front paws touch wall)	5-9	3	3	3	3	3
	10+	4	4	4	4	4
Checked signs*:						
** use severity scale: 1= present/mild, 2 = moderate, 3 = intense*						
Face grooming (moving paws across face)		2	2	2	2	2
Defecation/diarrhea		3	3	3	3	3
Abnormal Posture		3	3	3	3	3
Ptosis		2	2	2	2	2
Irritability/vocalization (spontaneous or touch)		3	3	3	3	3
Locomotion/Forepaw treading		1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Total Points (of 32 possible)						

Appendix C: Naloxone-Precipitated Withdrawal Score Sheet for Adolescents (PND 46-48)

	Rat ID						
	Nal-Inj.Time						
Withdrawal Sign		**Points**	**Points**	**Points**	**Points**	**Points**	**Points**
Body weight loss in 60 min	(1 per gram lost)						
Graded signs:							
Wet dog shakes	1-2	2	2	2	2	2	2
	3+	4	4	4	4	4	4
Front paw tremors	1-2	2	2	2	2	2	2
	3+	4	4	4	4	4	4
Jump attempts	2-4	2	2	2	2	2	2
	5-9	3	3	3	3	3	3
	10+	4	4	4	4	4	4
Checked signs:							
Abdominal constrictions/spasms		2	2	2	2	2	2
Genital grooming/penile erection		3	3	3	3	3	3
Teeth chattering		2	2	2	2	2	2
Irritability/vocalization upon touch		3	3	3	3	3	3
Swallowing movements		2	2	2	2	2	2
Ptosis (drooping eyes)		2	2	2	2	2	2
Abnormal posture		3	3	3	3	3	3
Defecation/diarrhea		3	3	3	3	3	3
Profuse salivation		7	7	7	7	7	7
	Total Points						
	Rater Initials						

VITA

PARKER BARNES

Education:	M.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2024
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