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Gestational buprenorphine exposure: Effects on pregnancy, development, neonatal opioid withdrawal syndrome, and behavior in a translational rodent model

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ABSTRACT

Background: The opioid crisis has led to an increased number of pregnant opioid-dependent women receiving opioid-maintenance therapy (e.g. buprenorphine, BUP), but little is known about the consequences of gestational BUP exposure on pregnancy outcomes, maternal care, or offspring development.

Methods: Our translational rodent model began BUP exposure to adult female rats (N = 30) at least 7 days before conception and continued throughout the postpartum period. Both *therapeutic* low-dose (BUP-LD, 0.3 mg/kg, s.c.) and *overexposure* high-dose (BUP-HD, 1.0 mg/kg) doses of BUP were compared to saline control. Female rats were bred in house with drug-naïve adult male rats. The day after parturition, litters were culled to 5 males/5 females and assigned randomly to various behavioral tests and assessed either neonates or adolescents. Litter characteristics, maternal caregiving, Neonatal Opioid Withdrawal Syndrome (NOWS), offspring development and adolescent behaviors were evaluated.

Results: BUP-LD decreased maternal care, delayed offspring development, decreased offspring body weight, length, temperature, and pain sensitivity (p's < .05). BUP-HD drastically reduced maternal care and offspring survival, altered litter characteristics, and increased NOWS (p's < .05).

Conclusion: These results demonstrate that the *therapeutic* BUP-LD in rats was relatively safe with subtle effects on maternal care and rodent offspring. However, *overexposure* BUP-HD in rats produced NOWS and compromised maternal caregiving as well as rodent offspring survival. More research is critical to validate the translational implication of these findings for human opioid-dependent mothers maintained on BUP-maintenance therapy.

1. Introduction

Decades of increased access to prescribed and illicit opioids has produced an international opioid epidemic (Humphreys, 2017; Kolodny et al., 2015; SAMHSA, 2017). Unsurprisingly, opioids are prescribed regularly to women of reproductive ages (Yazdy et al., 2015). SAMHSA reported that 7.5% of women (ages 18–25) misused opioid prescriptions in 2016 (Ailes et al., 2015; SAMHSA, 2017). Another study of ~1.1 million women on Medicaid reported 21.6% filled an opioid prescription during pregnancy (Desai et al., 2014). Between 2000–2012, gestational opioid exposure led to a five-fold increase in infants diagnosed with Neonatal Abstinence Syndrome (NAS) (FAD, 2016), also known as Neonatal Opioid Withdrawal Syndrome (NOWS) (Sutter et al., 2014), necessitating morphine treatment in up to 97% of exposed infants (Campbell, 2016; Patrick et al., 2012; Wiles et al., 2014). Ramifications of NAS/NOWS are severe because of dysregulation in the central and autonomic nervous systems (for review see Kocherlakota, 2014, 2019). Collectively, opioids during pregnancy raise significant concerns of adverse consequences for the mother, her pregnancy, and subsequent infant outcomes (Broussard et al., 2011; CSAT, 2006).

Opioid-maintenance therapies are prescribed regularly to treat opioid-dependent expectant mothers (WHO, 2014; Zedler et al., 2016) despite shortcomings in understanding gestational opioid misuse, opioid-maintenance therapy, and long-term infant outcomes (Laslo et al., 2017). The long-term effects of opioid-maintenance therapies

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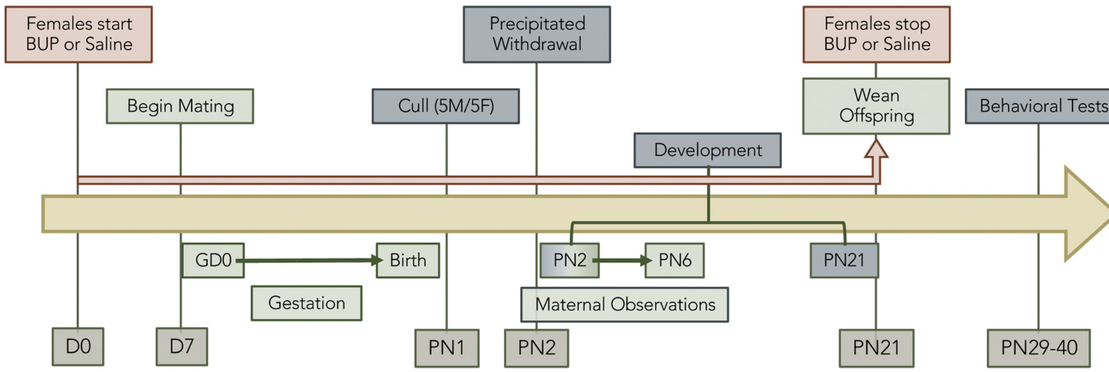

Fig. 1. Schematic of experimental timeline.

(e.g., buprenorphine or methadone) are preferred to the more severe consequences of misused opioids (Kakko et al., 2008; Yazdy et al., 2015). The semi-synthetic opioid buprenorphine (BUP) is less likely than methadone to produce respiratory depression or have adverse effects on the maternal hypothalamic-pituitary-adrenal axis (D'Elia et al., 2003; Davis, 2012). Because BUP is less lipophilic than synthetic opioids, it does not cross the placenta as readily (Szeto, 1993) and has an extended mechanism of action as well as half-life (Barrett et al., 1993; Kocherlakota, 2019). Accordingly, BUP is a favorable option for pregnant opioid-dependent women (Caritis et al., 2017; Kampman and Jarvis, 2015).

Cases of BUP maintenance in humans report reduced negative effects on infants as opposed to unmaintained and/or methadone-maintained pregnancies (Kocherlakota, 2019). Compared to infants born to mothers maintained on methadone, infants born to mothers on BUP tend to have less severe NAS/NOWS, are less likely to be born preterm, have higher birth weights, larger head circumference, and shorter hospital stays (Brogly et al., 2014; Hall et al., 2018; Metz et al., 2011; Zedler et al., 2016). Despite these relatively favorable outcomes, there are still discrepancies in preclinical findings regarding adverse consequences of gestational BUP exposure.

Preclinical models can help disentangle life-long neurobehavioral and physiological effects of *in utero* BUP exposure. However, some of these studies report no developmental impact on offspring, while others report decreased birth weight, increased pup mortality, increased depressive-like behavior, and oxidative stress (Barron and Chung, 1997; Chiang et al., 2010; Hung et al., 2013; Hutchings et al., 1996; Robinson, 2000, 2002; Sanchez et al., 2008; Wu et al., 2014). Further, long-term effects on endogenous opioid systems due to *in utero* opioid exposure are also indicated by increased nociception, analgesic tolerance, and increased reward sensitization (Chiang et al., 2010; Hou et al., 2004; Vassoler et al., 2016). A recent review of gestational opioid exposure in animal models showed that most preclinical models begin opioid administration (e.g., methadone or BUP) only *after* conception and stop *before* birth, thus limiting exposure to a period likely to be rare clinically (Byrnes and Vassoler, 2017). Of critical concern, previous models have not accounted for the fact that women are often already using opioids at the time of conception and continue use throughout parturition and postpartum periods. Models that begin drug exposure during pregnancy and stop before parturition cannot distinguish effects mediated by the maternal response to initiation of drug use within pregnancy from opioid exposure itself (Pechnick, 1993), nor can they account for influences of BUP exposure on the mother-infant bond postpartum (Swain et al., 2019).

Our translational animal model aimed to resemble human patterns of exposure in typical pregnant women undergoing BUP maintenance therapy. Adult female rats were exposed to either a saline control (SAL), a *therapeutic* low-dose (BUP-LD), or *overexposure* high-dose (BUP-HD) of BUP starting at least 7 days before conception. Treatment continued throughout gestation and postpartum until offspring were separated from dams. After parturition, the occurrence of NOWS and maturational development were evaluated in offspring. Post-weaning, male and female adolescent offspring were tested for differences in behavioral and cognitive outcomes. Overall, our results suggest that *therapeutic* BUP-LD had subtle consequences on dams and offspring, while *overexposure* BUP-HD elicited NOWS and drastically decreased maternal caregiving behavior as well as offspring survival.

2. Methods

2.1. Gestational buprenorphine exposure

Male (n = 10) and female (n = 30) Sprague-Dawley rats (~200–250 g) purchased from Charles River Laboratories (Wilmington, MA) were housed by sex (2–3 per cage) in a 12-h light/dark cycle (lights on at 07:00 h) with food and water available *ad libitum*. After 3 days of habituation/handling, females received daily injections of either saline (n = 10), low- (n = 10), or a high-dose (n = 10) BUP that continued until the end of postpartum at postnatal day 21 (PN21). Seven days after the start of injections, females were housed with a male each night until the detection of sperm presence via lavage sample confirmed conception, defined as gestation day 0 (GD0). Females were housed individually throughout gestation and after delivery (until PN21). All procedures were approved by the Wayne State University Institutional Animal Care and Use Committee (Protocol #17-11-0427) and held to the principals and guidelines established by the National Institute of Health for preclinical research (NIH, 2015), complied with the ARRIVE guidelines (Kilkenny et al., 2010), and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 (Hollands, 1986). For an experimental timeline, see Fig. 1.

2.2. Buprenorphine exposure

BUP (Sigma-Aldrich #B9275) was dissolved into sterile saline for final concentrations of 0.3 or 1.0 mg/ml BUP. Females were assigned randomly to 1 of 3 treatment groups: saline-vehicle (SAL), low-dose BUP (0.3 mg/kg, "BUP-LD"), or high-dose BUP (1.0 mg/kg, "BUP-HD"). The low-dose BUP was selected to mimic *therapeutic* doses comparable to what is prescribed for pregnant opioid-dependent women (Hung et al., 2013) and the high-dose was selected to imitate an *overexposure* level (Robinson and Wallace, 2001; Sanchez et al., 2008; Wu et al., 2014) as women may relapse despite being on opioid maintenance therapy and thus experience higher levels of opioids than prescribed (Swain et al., 2019). BUP and SAL were administered subcutaneously (s.c.) once daily between 08:00-09:00 h in a volume of 1.0 ml/kg. Dams were weighed every 3–4 days and drug volume was continuously adjusted from preconception through postpartum.

2.3. Birth measures

The day of parturition was designated postnatal day 0 (PN0). Deceased pups and unconsumed placentae were recorded and removed from the cage. Pups were examined on PN1 for obvious morphological anomalies, sexed by relative anogenital distance, and culled pseudo-randomly to n = 10 pups (random except for keeping an equal number of male and female pups per litter when possible). Gestation length, litter size, sex ratio (percent males), weight, temperature, and the number of deceased offspring were assessed. Neonatal deaths were noted from PN1 through PN3. Two males and two females per litter were used for evaluation of NOWS on PN2. One male and one female per litter were sacrificed on PN7 and their brains removed for future analysis. The remaining two males and two females were used for behavioral tests beginning PN29. Litters remained with their biological mothers and offspring maturation was assessed periodically between PN1-21.

2.4. Maternal observations

Maternal care of litters was observed for 5 days (PN2-6) and scored in one-min bins during three separate 20-min sessions (between 09:00-14:00 h) by a researcher blind to treatment condition (Myers et al., 1989). Behaviors were coded as time spent either (1) nursing, licking, grooming, (2) self-grooming, or (3) off-nest. If a dam neglected to care for her pups, they were removed from her cage at end-of-day PN2 and given to a SAL dam to ensure survival (see 3.1 *Maternal and Litter Results*). Following the last observation on PN2, a pup retrieval test was conducted where pups were placed across the cage at approximately equal distances outside the nest (Hahn and Lavooy, 2005). An experimenter blind to the treatment condition counted how many pups each dam returned to her nest during a 10-min assessment period.

2.5. Development and behavioral assessment

2.5.1. Spontaneous and precipitated withdrawal

Symptoms of NOWS were assessed in two males and two females per litter on PN2 via precipitated withdrawal using an opioid antagonist, naloxone (NLX) (Resnick et al., 1977). The severity of NOWS was evaluated by comparing symptoms between NLX-challenge (1 male/1 female) against a spontaneous-withdrawal control group (1 male/1 female) within each litter. NLX (2 mg/kg, Tocris Bioscience; CAS 357-08-4) was injected (s.c.) from a 0.5 mg/ml solution, while spontaneous-withdrawal (control) challenge pups were injected (s.c.) with 1.0 ml/kg saline. Immediately after injection, pups were placed in a warmed observation chamber for 30 min and behavior was observed, videotaped, and scored by two trained experimenters blinded to treatment condition. Withdrawal-related behaviors were scored continuously (for all 30-min) as hyperactivity, vocalization, mouth movements, stretching, face washing, and/or tremor (see supplementary materials Table S1 for definitions). After NOWS testing, all four animals were sacrificed.

2.5.2. Maturation

Offspring growth, development, and maturation were assessed from PN1-21 using standard behavioral teratology screening measures (Adams, 1986; Bowen et al., 2005). Maturation markers (i.e., surface righting, negative geotaxis, incisor eruption, eye opening, pinnae unfolding, as well as body and foot length) were selected from previous reports (for explanation see Bowen et al., 2005). Body weight and temperature were recorded each day and body lengths were measured on PN21.

2.6. Early-adolescent behavioral assessment

During early-adolescence (PN29-43), two males and two females from each litter were evaluated for behavioral and cognitive effects. One male and female from each litter were evaluated for anxiety-like behavior using the Elevated-Zero Maze. These same animals were then assessed for stress responses during Physical Restraint 3 days later. The other male and female from each litter were subjected to Hot-Plate testing and assessed for pain sensitivity under baseline and morphine-challenge conditions.

2.6.1. Elevated-zero maze

The Elevated-Zero Maze (EZM; Med Associates, Inc., Fairfax, VT) is a more sensitive variation of the Elevated-Plus Maze used to evaluate anxiety-like behavior in animals (Carobrez and Bertoglio, 2005; Shepherd et al., 1994; Zimprich et al., 2014). Male and female offspring (PN29-31) were placed individually at the same initial point (closed arm entrance area) and allowed to explore the EZM for 5-min. Trials were videotaped and analyzed with a behavioral tracking software (Noldus Ethovision, V.13) for time spent and distance traveled in the open and/or closed areas. Anxiety-like behaviors are indicated by immobility and/or spending more time in closed areas than open areas (Braun et al., 2011; Rodgers and Dalvi, 1997). After completion, offspring were returned to home cages.

2.6.2. Restraint stress sensitivity and corticosterone analysis

Three days after EZM testing, the same male and female offspring (PN32-34) were placed into a clear acrylic physical restrainer (Plas Labs™ Broome-Style Rodent Restrainers, 551BSRR) for 30 min to induce a stress response. Blood was collected from the saphenous vein to analyze corticosterone (CORT) response as previously described by Kott et al. (2019). Three blood samples were collected at baseline (Time-1, before the stressor), peak (Time-2, immediately after the stressor), and recovery (Time-3, 90 min post stressor) within 3 min of touching each animal. Blood samples clotted for 1-h before serum was extracted after centrifugation at 8000 G then analyzed for CORT response levels using ELISA assays (Ann Arbor Assays, K-14 H) according to manufacturer instruction. All samples were run in duplicate and intra- and inter-assay coefficients were less than 9.5%.

2.6.3. Pain sensitivity

Pain threshold and analgesic efficacy were evaluated using the Hot-Plate (Barrot, 2012; Deuis et al., 2017; Woolfe and MacDonald, 1944). Male and female offspring (PN29-31) were acclimated to the Hot-Plate one day prior to baseline testing to limit novelty stress. On baseline test day (PN30-32), each animal was placed individually on the Hot-Plate instrument (50 °C) surrounded by a Plexiglas wall. The latency to display a nociceptive response was recorded (fore/hind paw withdraw/licking or paw stamping) (Espejo and Mir, 1993). To prevent tissue damage, rats were immediately removed after the first sign of a nociceptive response (cut off: 60-sec). One week later (PN37-39), animals were administered an active dose of morphine (5 mg/kg, s.c., NIH/NIDA) 60-min prior to the Hot-Plate re-test (morphine challenge) as previously validated (Jøhannesson and Becker, 1973).

2.7. Data analysis

Body weight, litter characteristics, and maternal care behaviors were analyzed with repeated measures (RM) ANOVA or multivariate ANOVA (MANOVA) with SAL, BUP-LD and BUP-HD as between subject factors. NOWS was evaluated by MANOVA with naloxone-challenge and saline-control as between subject factors within SAL, BUP-LD and BUP-HD groups. Offspring body weight, temperature, maturation, anxiety-, stress-, and pain-sensitivity were analyzed with either RM ANOVAs or MANOVA with SAL and BUP-LD as between subject factors. Weight was recorded every 3–4 days (PN1-40) and mean body temperature was calculated every 3 days from PN1-30. The Greenhouse-Geisser correction was reported. Tukey's *post-hoc* analyses were conducted when appropriate. All data were analyzed with SPSS version 25 and results were considered significant if $p < .05$. Non-significant

Table 1
Maternal and Litter Characteristics for SAL (0.0 mg/kg), BUP-LD (0.3 mg/kg) and BUP-HD (1.0 mg/kg) are displayed. # reflects BUP-HD is different from SAL, * indicates BUP-HD is different from BUP-LD, ** reflects BUP-HD is different from SAL and BUP-LD, ! indicates numbers after culling. *P*-values indicate between group effects, n.s. indicates non-significant, n.a. indicates not analyzed.

Level of BUP	0.0 mg/kg			0.3 mg/kg			1.0 mg/kg			*p*
Number of dams (N = 30)	10			10			10			n.s.
Number of litters born (N = 26)	8			8			10			n.s.
Live litters post PN2**	8			7			6			0.025
Litters surrogate-fostered**	0			1			4			0.034
Litters compared after PN2	8			6			0			n.a.
Length of gestation (days)	21.75	±	0.16	21.86	±	0.14	22.10	±	0.18	n.s.
Number of placenta found#	0.38	±	0.38	0.88	±	0.52	3.90	±	1.18	0.018
Mean litter size	14.50	±	0.73	14.86	±	0.67	12.90	±	0.84	n.s.
Number male	6.50	±	1.05	6.14	±	0.83	6.60	±	0.72	n.s.
Number female*	7.88	±	0.92	8.29	±	0.52	5.60	±	0.54	0.018
Sex Ratio	45%	±	0.23	41%	±	0.13	51%	±	0.20	n.s.
Number of pups alive PN0**	14.37	±	0.73	14.29	±	0.57	11.00	±	1.10	0.018
Number of pups alive PN1**	14.37	±	0.73	13.71	±	0.68	8.80	±	1.23	0.001
Number of pups alive PN2#!	10.00	±	0.00	9.86	±	0.14	6.50	±	1.40	0.025

effects were not discussed.

3. Results

3.1. Maternal and litter results

Four of the 30 treated females were determined not to be pregnant despite the presence of sperm. Since we did not identify uterine implantation sites, we cannot know if the SAL (n = 2) or BUP-LD (n = 2) failed pregnancies were due to false positive of sperm identification or spontaneous loss of pregnancy (Kelty and Hulse, 2017). One BUP-LD dam was determined to be an outlier (± 2 SDs) as she delivered her pups (n = 5, 1 stillborn) significantly early (GD18) and was excluded from analysis. Further, there were unforeseen complications within the BUP-HD group that resulted in the exclusion of all high-dose pups from this study after PN2. Four BUP-HD litters did not survive beyond PN2. Mortality reports included both stillborn offspring as well as any offspring deaths within 48 h of birth after culling litters to 5 males/5 females (see Table 1 and Fig. 2).

A majority of BUP-HD dams failed to engage in basic maternal care (e.g., nursing). In an effort to promote pup survival, we chose to give affected BUP-HD litters to control dams on PN2 (16:00 h); all surrogate-fostered offspring survived. Because a majority of these BUP-HD pups: (1) experienced maternal neglect and potential malnutrition for up to 48 h, and (2) were confounded due to surrogate-fostering, these dams/offspring were excluded from analyses after PN2. While some BUP-HD dams (n = 2) did engage in normal maternal care and remained with their litters, the sample size was too small to be included in further analyses. Therefore, development and behavior outcomes focus exclusively on SAL and BUP-LD groups.

3.1.1. Litter characteristics

MANOVA analysis determined a significant effect of BUP on litter outcomes [$F(20,26) = 3.192$, $p = .003$], see Table 1. There were main effects for number of placenta found [$F(2,22) = 4.810$, $p = .018$], number of surviving offspring on PN0 [$F(2,22) = 4.849$, $p = .018$], PN1 [$F(2,22) = 9.998$, $p = .001$], and PN2 [$F(2,22) = 4.391$, $p = .025$] and number of females born [$F(2,22) = 4.823$, $p = .018$]. BUP-HD, but not BUP-LD, had an increased number of placenta found compared to SAL (Tukey post-hoc: $p < .05$), see Fig. 2A. BUP-HD exposure resulted in lower number of surviving offspring as compared to both SAL and BUP-LD on PN0 and PN1, as well as a lower number of surviving offspring as compared to SAL on PN2 (Tukey's *post-hoc*: p's $< .05$). See Fig. 2B for the number of deceased pups per litter. Finally, BUP-HD had a reduced number of females as compared to BUP-LD (Tukey's *post-hoc*: $p < .05$).

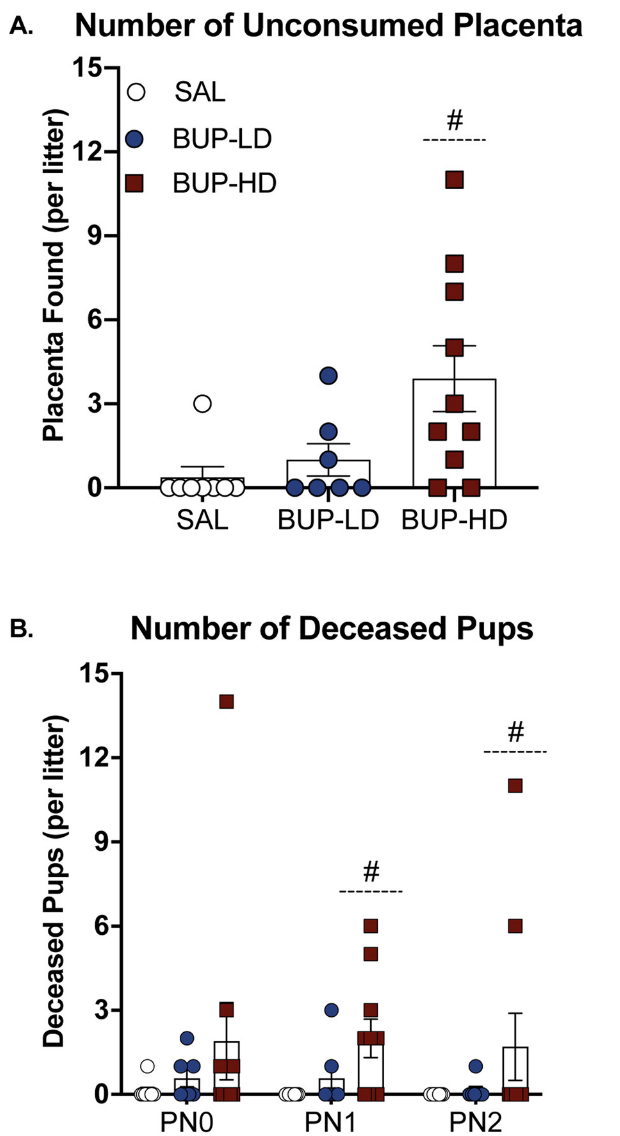

Fig. 2. A–B. The effects of gestational BUP exposure on number of unconsumed placenta post parturition (A) and number of deceased pups found on PN0, PN1, and PN2 (B). Data points reflect each dams' individual score. # BUP-HD group is different from SAL group ($p < .05$).

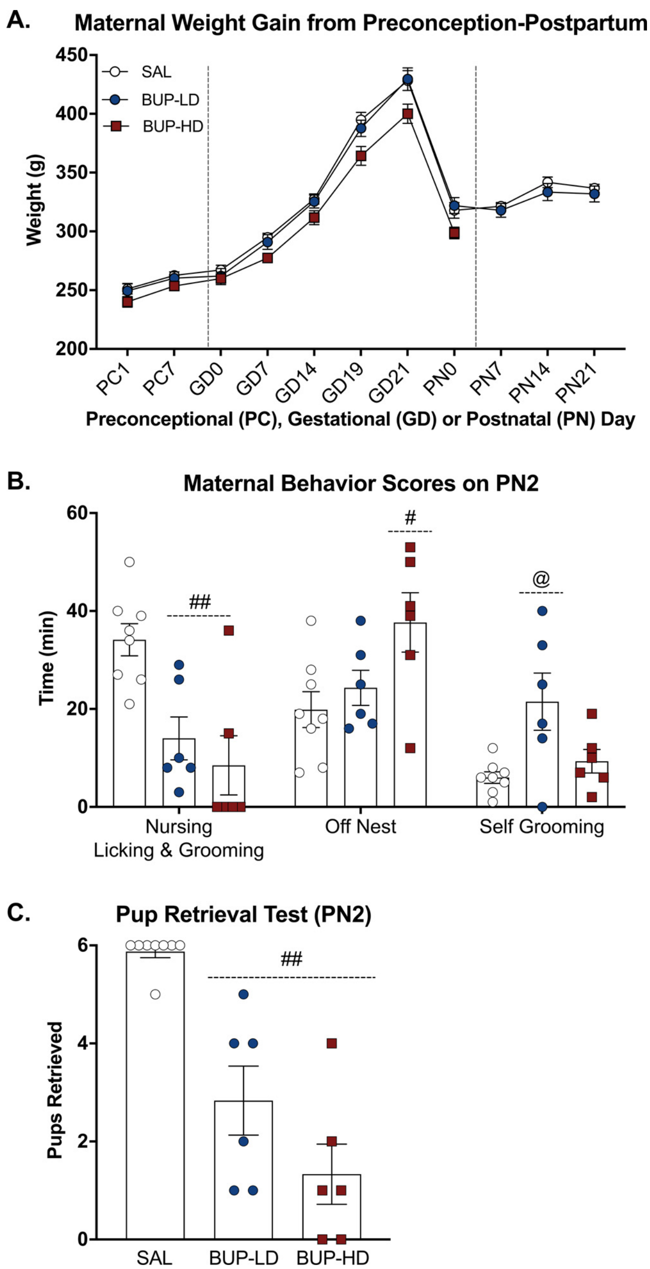

Fig. 3. A–C. The effects of gestational BUP exposure on female weight gain during preconception, gestation, and postpartum periods (A), maternal behavior on PN2 (B), and pup retrieval on PN2 between SAL, BUP-LD and BUP-HD groups. (A) Values reflect mean (± S.E.M.), (B&C) values in reflect individual scores per dam. ## BUP-HD and BUP-LD groups are different from SAL group, # BUP-HD group is different from SAL group, @ BUP-LD group is different from SAL and BUP-HD groups ($p < .05$).

3.1.2. Maternal body weight

Maternal weight was analyzed by RM ANOVA for the preconception, gestation and postpartum periods (see Fig. 3A). There was no significant effect of BUP on preconceptional body weight (p > .05). During gestation, dams were weighed on GD0, 7, 14, 19, 21, and PN0. A significant covariate of Litter Size [F(1, 21) = 6.207, p = .021] rendered the main effect of BUP nonsignificant (p > .05). There was a main effect of Gestation Day, [F(3.603, 75.673) = 6.878, p < .001] reflecting weight gain during gestation and a significant interaction of Gestation Day x Litter Size [F(3.603, 75.673) = 5.475, p = .001], consistent with larger litter sizes contributing to greater maternal weight gain. Postpartum weight was analyzed on PN7, PN14, and PN21 between SAL and BUP-LD groups only. There was a main effect of Postpartum Day [F(3, 39) = 14.124, p < .001] indicating dams gained weight post parturition.

3.2. Maternal Behavior

3.2.1. Maternal care

Univariate ANOVA demonstrated a significant effect of BUP for all 3 groups (SAL, BUP-LD, BUP-HD) on PN2 maternal behavior [F(2,17) = 5.565, p = .014]. BUP-LD/HD engaged in less nursing/licking/grooming than SAL, BUP-LD engaged in more time self-grooming than SAL, and BUP-HD engaged in more time off nest than SAL (Tukey post-hoc: p's < 0.05); see Fig. 3b. Maternal behavior differences on PN3-PN6 also showed significant reductions in BUP-LD maternal care as compared to SAL dams and are displayed in supplementary materials Fig. S1.

3.2.2. Pup retrieval

There was also a significant effect of BUP in the total number of pups (max: n = 6 per litter) returned to the nest on PN2 as determined by univariate ANOVA [F(316) = 23.973, p < .001], see Fig. 3C. BUP-LD/HD dams retrieved fewer pups than SAL dams (Tukey's post-hoc: p's < 0.025) with no difference between BUP-LD and BUP-HD dams.

3.3. Development and behavioral analyses

3.3.1. Spontaneous and precipitated withdrawal

MANOVA analysis demonstrated a significant effect of BUP [F(12,163) = 22.814, p = .002] on NOWS symptoms in all 3 groups for spontaneous (n = 48) and naloxone (NLX) (n = 50) precipitated withdrawal, see Fig. 4. BUP-HD offspring had increased hyperactivity, vocalizations, and stretching as compared to SAL and BUP-LD offspring (Tukey's post-hoc: p's < 0.025). Additionally, BUP-HD offspring had increased mouth movements and face washing as compared to SAL (Tukey's post-hoc: p's < .020); see Fig. 4A-E. There was a significant multivariate effect of NLX [F(6,81) = 2.741, p = .018] on all exposure groups. Forelimb tremors decreased in the NLX challenge condition as compared to spontaneous withdrawal (p = .017); see Fig. 4f.

3.3.2. Offspring weight gain

RM ANOVA assessed total body weight between all 3 groups (SAL, BUP-LD, BUP-HD) on PN1-2. There was a main effect of BUP [F(2, 205) = 32.353, p < .001] such that SAL pups were heavier than BUP-LD and BUP-HD pups at PN1 and PN2, with no significant difference between BUP-LD and BUP-HD pups (Tukey post-hoc: p's < 0.001), see Fig. 5A inset. There was a main effect of Sex [F(1, 205) = 14.683, p < .001] with males weighing more than females (p < .05). There was a significant main effect of Postnatal Day [F(1,205) = 81.677, p < .001] with all groups weighing more on PN2 than PN1 (Tukey's post-hoc: p's < 0.05).

Because BUP-HD was excluded from analysis after PN2 (see 3.1: *Maternal and Litter Results*), a separate RM ANOVA evaluated total body weight for the SAL and BUP-LD groups during pre- and post-weaning periods (PN1-21 and PN22-40). There was a significant main effect of BUP-LD from PN1-21 [F(1, 51) = 8.705, p = .005], with BUP-LD weighing less than SAL offspring, and a main effect of Postnatal Day [*F*(1.759, 89.705) = 4514.711, $p < .001$] confirming offspring gained weight (p's < .05), Fig. 5A. There was no effect of BUP-LD post-weaning (PN22-40). However, there were significant effects of Sex [*F*(1, 51) = 59.296, $p < .001$] and Postweaning Day [*F*(1.336, 68.145) = 4128.050, $p < .001$] as well as Postweaning Day x Sex interaction [F(1.336, 68.145) = 130.960, p < .001], confirming males were heavier than females and gained significantly more weight as they aged (p's < .05, data not shown).

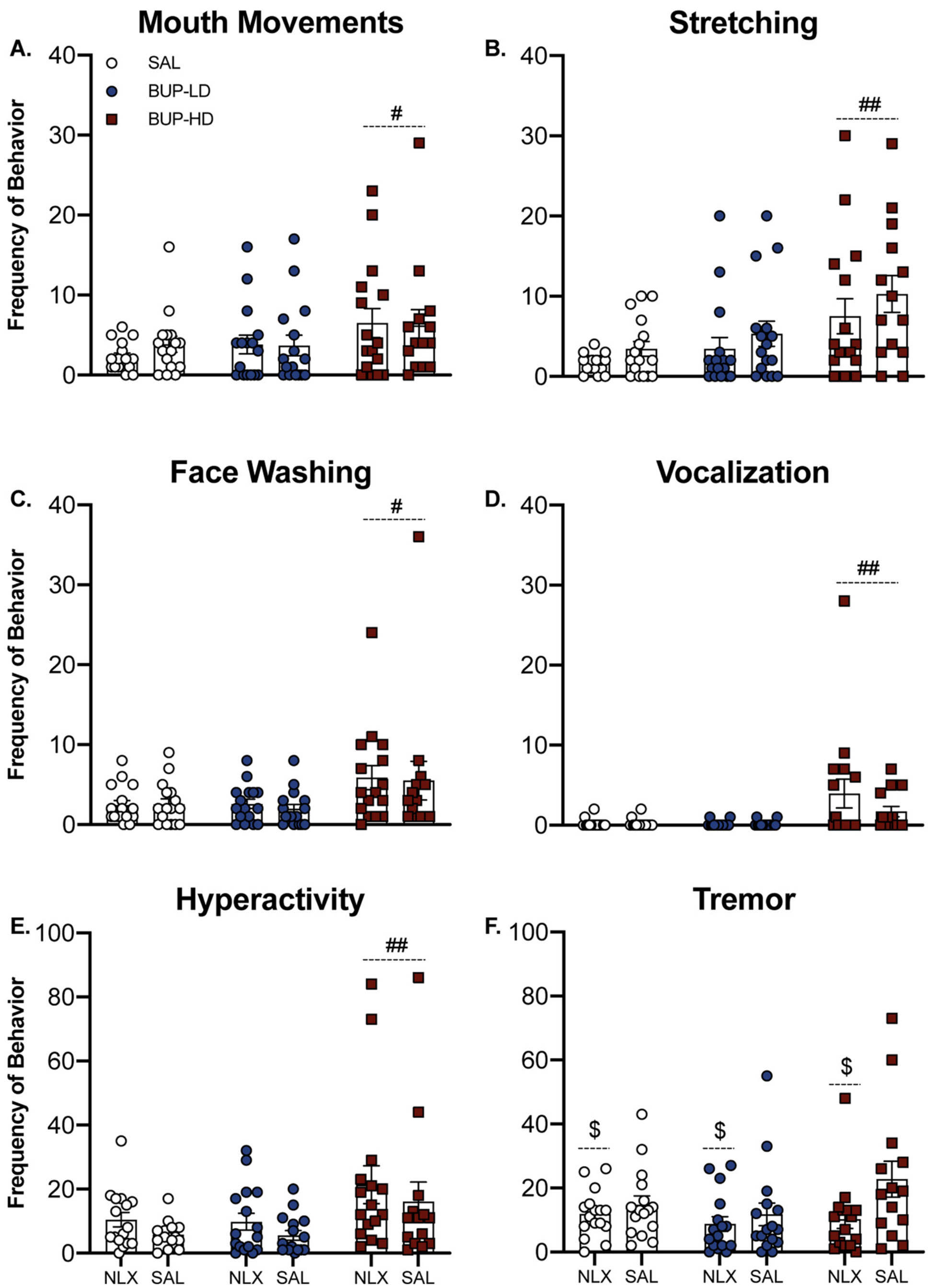

Fig. 4. A–F. The effects of gestational BUP exposure on symptoms of Neonatal Opioid Withdrawal Syndrome displayed as mouth movement (A), stretching (B), face washing (C), vocalization (D), hyperactivity (E) and tremor (F) on PN2. Data points shown reflect individual scores per pup. # BUP-HD is different from SAL, ## BUP-HD is different from SAL and BUP-LD, $ Naloxone (NLX) is different from Saline-control (SAL) ($p < .05$).

3.3.3. Offspring biobehavioral development

MANOVA analysis showed a significant multivariate effect of BUP-LD [F(7, 45) = 3.875, p = .002] on negative geotaxis, incisor eruption, eye opening, pinnae unfolding, and body length; BUP-LD had delayed development compared to SAL offspring. There was no effect of BUP-LD on surface righting or foot length (p's > 0.05); see Fig. 5B.

3.3.4. Offspring body temperature

RM ANOVA demonstrated a significant main effect of BUP-LD [F(1, 55) = 4.436, p = .040] and Postnatal Day [F(5.313, 292.228) = 11.644, p < .001] revealing BUP-LD pups had lower body temperatures than SAL pups overall (Tukey post-hoc: p's < 0.05 on days PN1-3 and PN10-12), see Fig. 5C. The interaction of Postnatal Day x BUP-LD was trending (p = .053), but there were no effects or interactions with Sex (p's > 0.05).

3.4. Early-adolescent behavioral assessment

3.4.1. Anxiety-like behavior (Elevated-Zero Maze)

MANOVA analysis revealed no significant effect of BUP-LD on time spent in open arms or time spent moving as compared to SAL adolescent offspring (p's > .05). There was no effect or interaction with Sex (p's > .05) on any EZM behaviors; see supplementary materials Fig. S2A.

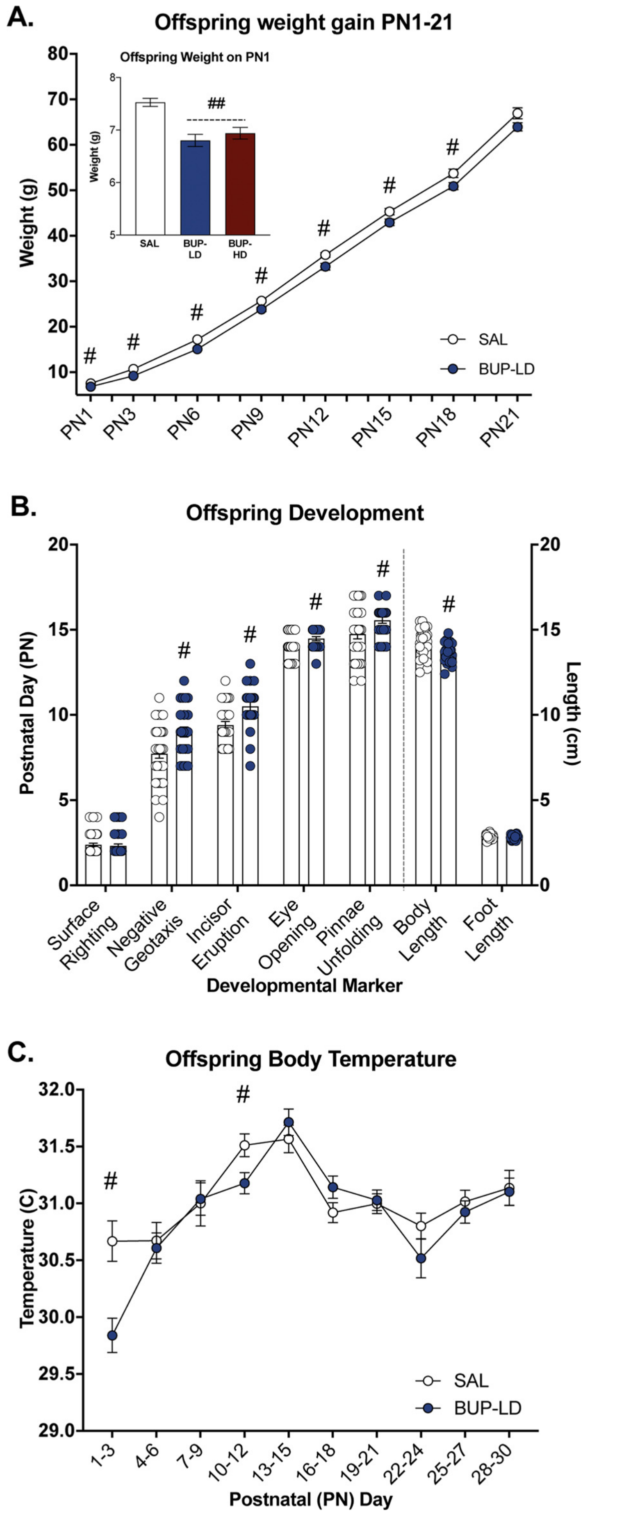

Fig. 5. A–C. The effects of gestational BUP exposure offspring weight gain from PN1-21 (A), offspring development (B), and offspring body temperature (C). (A &C) Values show mean (± S.E.M.) of pup weight and body temperature between BUP-LD and SAL groups, inset graph shows mean (± S.E.M.) of pup weight between SAL, BUP-LD and BUP-HD groups on PN1, (B) Data points show individual scores per pup tested between BUP-LD and SAL groups. ## BUP-HD and BUP-LD group is significantly different from SAL group, # BUP-LD group is significantly different from SAL group ($p < .05$).

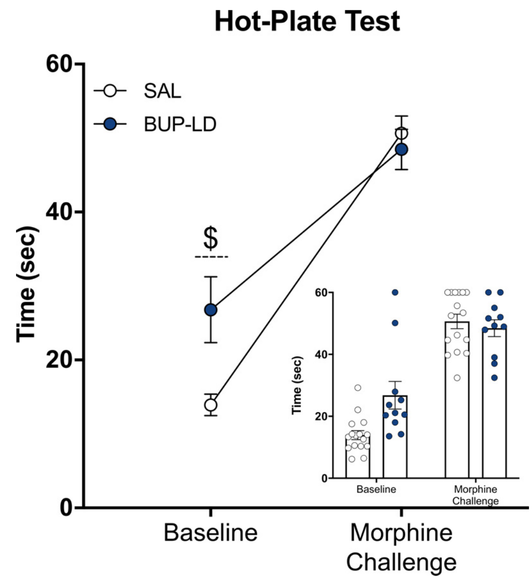

Fig. 6. The effects of gestational BUP exposure on latency to display a nociceptive response during baseline and morphine challenge conditions between SAL and BUP-LD groups. Shown are mean (± S.E.M.) of latency to withdrawal a paw from a heat stimulus. Inset graph shows individual scores per pup tested. $ reflects an interaction of morphine and BUP-LD, with post hoc revealing a between group difference at baseline.

3.4.2. Stress response (physical restraint)

RM ANOVA found no effect of BUP-LD or Sex (p's > .05) on stress response to physical restraint at Time-1 (baseline), Time-2 (peak), or Time-3 (recovery). There was a significant difference in CORT response between Time Point [F(1.241, 29.772) = 31.470, p < .001], with all animals showing the expected rise in CORT at Time-2 (pairwise comparisons: Time-2 significantly higher than Time-1 and Time-3 (p's < .001)), but no interaction between Time Point x BUP-LD (p > .05); see supplementary materials Fig. S2B.

3.4.3. Pain-Sensitivity (Hot-Plate)

RM ANOVA did not identify a main effect of BUP-LD on latency to display a nociceptive behavior ($p > .05$). However, there was a within subject effect of Morphine [$F(1, 23) = 145.884$, $p < .001$] which increased withdrawal latency in both groups. There was also a significant interaction of BUP x Morphine [$F(1, 23) = 9.582$, $p = .005$] which confirmed BUP-LD adolescents displayed an increased latency to withdrawal under baseline conditions as compared to SAL (Pairwise comparison: $p < .05$), but there was no difference between groups in time to withdrawal during the morphine challenge (Pairwise comparison: $p > .05$), see Fig. 6.

4. Discussion

The rationale for this study is the drastic increase of opioid use during pregnancy which is often treated using opioid-maintenance therapy like buprenorphine (BUP). The present work found significant dose-dependent effects of gestational BUP exposure from preconception through postpartum periods in both dams and offspring. The high-dose BUP (BUP-HD) exposure decreased number of females born, maternal care, and pup body weight (PN1-2) and increased pup mortality. Further, surviving BUP-HD pups showed signs of Neonatal Opioid Withdrawal Syndrome (NOWS) on PN2. The low-dose BUP (BUP-LD) exposure resulted in decreased maternal care, offspring body weight (PN1-21), body length, body temperature, delayed development, and reduced pain sensitivity in adolescence. Interestingly, BUP exposure did not substantially impact maternal weight gain, offspring anxiety-like behavior or stress-sensitivity. Overall, our results suggest that BUP has dose-dependent consequences for the dam and her offspring with BUP-HD having more detrimental effects than BUP-LD.

4.1. Maternal and litter characteristics

We found no significant effect of BUP exposure on gestational weight gain, in line with previous reports using similar dose ranges (e.g., 0.3–3.0 mg/kg) (Chen et al., 2015; Hutchings et al., 1995). Barron and Chung (1997) found dams exposed to BUP (0.3 and 0.6 mg/kg) showed a transient decrease in food and water intake during initial BUP exposure. Though we did not track food and water intake, by starting BUP exposure 7 days prior to mating, any initial changes in food or water intake would have been overcome before pregnancy (Swain et al., 2019).

Our BUP-HD dams gave birth to fewer females, consumed fewer placentas, and had significant pup mortality compared to SAL and BUP-LD dams (40% of BUP-HD litters died by PN2). Our results are unique in contrast to other studies of gestational BUP exposure where pup mortality occurred at 3.0 mg/kg, but not 1.0 mg/kg (Barron and Chung, 1997; Chen et al., 2015; Chiang et al., 2010; Hutchings et al., 1996, 1995). Because our novel translational model included continuous daily administration (s.c.) of BUP across gestational periods (not modeled before), the addition of pre- and post-natal BUP exposure periods may be responsible for our more drastic findings. In contrast, previous models have discontinued BUP treatment before parturition and cross-fostered pups at birth which does not carefully reflect human treatment patterns as human mothers are typically maintained on BUP throughout gestation, parturition, and postpartum periods.

There were clear signs of maternal neglect in the BUP-HD group that we believe contributed substantially to pup mortality. At parturition, BUP-HD had decreased placental consumption (poor placentophagia) which may have been another indicator of poor maternal care or an effect of aversion as BUP is identifiable in placenta (Concheiro et al., 2010). At the start of maternal care quantification (PN2), we noticed BUP-HD dams had a striking decrease in nursing/licking/grooming behavior, spent more time off the nest, and failed to retrieve and return pups to the nest as compared to SAL dams. BUP-LD dams also showed a decrease in nursing/licking/grooming behavior, spent more time self-grooming, and failed to retrieve and return pups to the nest, though to a less severe degree than BUP-HD dams. BUP-LD continued to show reduced maternal behavior as compared to SAL from PN3-6 which may have influenced subsequent pup maturation/behavior (see supplementary materials).

To promote offspring survival, BUP-HD litters were given to SAL dams at end-of-day PN2. This prevented further offspring mortality which suggested that post-birth mortality was indeed due to maternal neglect. This speculation was supported by 2 of our BUP-HD dams who did display appropriate maternal care (and did not have post-birth mortality). However, the unexpected need to surrogate-foster compromised the design of this study and limited interpretation of the differential effects of gestational BUP-HD exposure versus maternal neglect.

Maternal behavior after gestational BUP treatment has often not been reported (Byrnes and Vassoler, 2017; Farid et al., 2008). One report showed BUP (3.0 mg/kg) decreased maternal care and increased possible cannibalism (labeled "predatory behavior") but did not provide details on maternal behavior (Chen et al., 2015). We found evidence of cannibalism on PN1 (n = 1 BUP-HD dam) but are unaware of any prior evidence that gestational BUP exposure would compromise maternal care to the degree seen presently. Because endogenous opioids play an important role in the initiation of maternal caregiving (de Mello Cruz et al., 2010; Farid et al., 2008; Mann et al., 1991; Stafisso-Sandoz et al., 1998), postpartum BUP treatment could be suppressing lactation/maternal care and thus influencing maternal or predatory behaviors via the Maternal Behavior Neurocircuit (MBN) (Klein et al., 2014; Swain et al., 2019).

Our current data does not allow for conclusions about whether the preconceptional exposure or the continued exposure throughout parturition and the postpartum were the driving factor for the increased pup mortality and maternal neglect in the BUP-HD group. However, a review by (Fodor et al., 2014) has reported that similar opioid exposure regimens of morphine (prior to conception) had no effect on maternal behavior but induced long-term effects on rodent offspring (mortality was not reported). However, it was also stated that morphine treatment during lactation profoundly influenced the mother's behavior (Fodor et al., 2014). Conceivably, high levels of BUP at the time of conception and/or parturition in the present study interfered with the opioid-dependent initiation of the maternal brain network and subsequent maternal care behaviors as indicated by similar evaluations of gestational opioid exposure (Russell and Brunton, 2019). Considering BUP's unique agonist/antagonist effects at the *mu*- and *kappa*- receptors, and the important involvement these receptors have in maternal behaviors (Brunton, 2019; Swain et al., 2019), it is further feasible that BUP impacted the maternal network more drastically than previously studied (*mu*-agonist) opioids. However, the deleterious effects of BUP-HD may not be specific to BUP but rather a consequence of a high dose opioid exposure regimen during gestation as a variety of negative consequences for the dam and her offspring after gestational morphine or methadone exposure have been reported (Byrnes and Vassoler, 2017).

4.2. Neonatal and developmental period

Survived BUP-HD offspring displayed NOWS on PN2. Because NLX did not precipitate withdrawal beyond spontaneous withdrawal counterparts, it is conceivable that BUP-HD offspring were already in the process of eliminating BUP and NLX precipitation may have been compromised by a ceiling effect. To our knowledge, one study has previously precipitated withdrawal in rats following *in utero* BUP (0.3–3.0 mg/kg) exposure (Robinson and Wallace, 2001). Their NLX administration (on PN1) found low-dose (0.3 mg/kg) BUP offspring showed withdrawal symptoms while the higher doses of BUP (1.0 and 3.0 mg/kg) did not. Our results showed an opposite pattern whereas our BUP-LD group showed no difference from SAL offspring, while our BUP-HD did. Considering previous (successful) withdrawal precipitation on PN1, and BUP's long rodent half-life (6–8 hrs) (Gades et al., 2000), we expected NOWS symptoms would still be present on PN2. Evidence suggested that despite our calculations, BUP may have no longer been present in the BUP-LD offspring's system, but that it remained bioactive in BUP-HD pups on PN2.

We also found BUP-LD/HD exposure reduced body weight in the perinatal period (PN1-2) consistent with some (Barron and Chung, 1997; Robinson and Wallace, 2001), but not other studies, using a dose range of 0.3–3.0 mg/kg (Chen et al., 2015; Chiang et al., 2010; Hutchings et al., 1996, 1995). Our BUP-LD pups had reduced body weight during pre-weaning (PN1-21), but not post-weaning (PN22-40) periods, possibly due to the continued, though very low (Ilett et al., 2012), exposure to BUP through the maternal milk until weaning. However, we cannot exclusively determine if the consequences of BUP-LD identified during the pre-weaning period are due strictly to BUP delivered via lactation or to the cumulative effects of daily BUP exposure from preconception through postpartum periods. A majority of previous models used an osmotic mini-pump for constant dam dosing and limited exposure to gestation only (~GD7-20) (Barron and Chung, 1997). Comparing our results to studies using mini-pumps could be problematic because a consistent dose/volume regime could ultimately decrease a dam's exposure to BUP (due to gestational weight gain) and inadvertently attenuate potential fetal exposure (Hutchings et al., 1996). Further, we saw that BUP-LD exposure caused a persistent reduction in offspring body temperature. This is unique as thermoregulation following perinatal BUP has not been previously reported. Notably, *mu*-1 opioid receptor stimulation has been shown to decrease body temperature in neonatal rats (Colman and Miller, 2002) and perinatal methadone exposure has been shown to have persistent thermoregulatory effects (Thompson and Zagon, 1981).

In contrast to previous studies (as reviewed by Byrnes and Vassoler, 2017), we demonstrated that BUP-LD exposure delayed development. Robinson and Wallace (2001) reported *accelerated* attainment of surface righting in pups exposed to low (0.3 mg/kg) but not high (3.0 mg/kg) BUP doses. However, a majority of previous animal studies have failed to identify developmental effects which may be attributed to use of restricted BUP exposure and/or cross-fostering (Hutchings et al., 1996, 1995; Robinson and Wallace, 2001). We suggest even therapeutic doses of continuous daily administration (s.c.) BUP exposure can have effects on maturation, but we cannot rule out the effects of maternal care. Importantly, our results appear to be consistent with clinical studies that report shortened body length and smaller head circumference after gestational BUP exposure (Farid et al., 2008; Jansson et al., 2017; Kakko et al., 2008).

4.3. Early adolescent behavior

When tested in adolescence, our offspring showed no effect of BUP-LD on anxiety-like behavior in the Elevated-Zero Maze (EZM). However, Chen et al. (2015) assessed offspring after a higher (3.0 mg/kg) gestational BUP exposure dose and found early adulthood males and females displayed increased anxiety-like behavior in a light-dark transition task, but only females did so in an Elevated-Plus Maze. While there are not many studies of long-term effects of gestational BUP, there are studies identifying anxiety-like behavior in offspring following gestational methadone and/or morphine (Alizadeh et al., 2018; Byrnes et al., 2011; Byrnes, 2005; Torabi et al., 2017). Further, no effects of BUP-LD exposure were observed on stress-sensitivity in male or female offspring. We could not identify alternative investigations following gestational BUP exposure, but there are similar studies following gestational morphine exposure that show an enhanced stress response (e.g., increased CORT) later in life (Castellano and Ammassari-Teule, 1984; Rimanóczy et al., 2003; Šlamberová et al., 2004).

Our data revealed that exposure to BUP-LD delayed baseline nociceptive response time which suggests reduced pain sensitivity in males and females during adolescence. However, when challenged with morphine, there was no difference in latency to withdraw between BUP-LD and SAL offspring. Our findings differ from Chiang et al. (2010) who showed that *in utero* BUP exposure (3.0 mg/kg) decreased sensitivity to morphine's analgesic effects in rats (8–12 weeks). Additionally, hyperalgesia and increased tolerance to morphine have been reported following gestational morphine exposure (O'Callaghan and Holtzman, 1976, 1977; Tao et al., 2011; Vathy, 2002). Though the mechanisms of action of morphine and BUP differ in their efficacy at the *mu*-receptor, these findings collectively demonstrate that gestational opioid exposure can have diverging long-lasting effects on offspring pain response.

5. Conclusions

Our model of gestational BUP exposure from preconception to the end of the postpartum identified more severe consequences of BUP on the dam and offspring than previously reported. Because we aimed to mimic human exposure patterns, our translational model suggests that BUP maintenance therapy beginning before conception and continuing throughout pregnancy and postpartum periods has more severe consequences on maternal behavior and offspring outcomes than previously indicated (Byrnes and Vassoler, 2017; Clancy et al., 2007). Though the profound impact of high-dose BUP on maternal neglect and subsequent pup mortality prevented systematic evaluation of BUP-HD effects after PN2, our study nevertheless provides evidence that BUP-HD exposure may have more serious risks than formerly realized.

Because opioid maintenance exposure and subsequent pharmacokinetics are not well validated for pregnant women (Zhang et al., 2018), the effects of repeated relapse (overexposure) or withdrawal during pregnancy are even less so understood and necessitate the use of clinically relevant translational models. Considering the ongoing international opioid crisis, there is dire need for explicit clinical and preclinical research on gestational opioid/maintenance exposure describing long-term health consequences for mothers and exposed offspring.

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None.

Contributors

CMW: Responsible for study design, handled controlled substances, treated all animals, conducted experiments and functional assessments, acquired and analyzed raw data, preformed statistical analyses, interpreted results, prepared table and figures, and wrote the first draft of the manuscript. SEB: Responsible for study design, handled controlled substances, advised data analysis and interpretation, edited tables and figures, and contributed significantly to manuscript edit and review. CLR: Contributed to study preparation, conducted experiments, and acquired/input data. LMR: Contributed to study preparation, conducted experiments, and acquired/input data. SB: Responsible for study design, advised experimental process as needed, advised data analysis and interpretation, edited tables and figures, and contributed significantly to manuscript edit and review.

All authors have read and approved the final manuscript.

Declaration of Competing Interest

We confirm that there were no conflicts of interest that impacted this study; no conflict declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2019.107625.

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