Enduring consequences of perinatal fentanyl exposure in mice

Jason B. Alipio[1]*, Adam T. Brockett[2,3]*, Megan E. Fox[1], Stephen S. Tennyson[2,3], Coreylyn A. deBettencourt[2], Dina El-Metwally[6], Nikolas A. Francis[4,5], Patrick O. Kanold[4,5], Mary Kay Lobo[1], Matthew R. Roesch[2,3], Asaf Keller[1]

1. Department of Anatomy & Neurobiology, Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD 21201
2. Department of Psychology, University of Maryland, College Park, MD 20742
3. Program in Neuroscience and Cognitive Science, University of Maryland, College Park, MD 20742
4. Department of Biology, University of Maryland, College Park, MD 20742
5. A. James Clark School of Engineering, Institute for Systems Research, University of Maryland, College Park, MD 20742
6. Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201

*** The first two authors contributed equally to this study**

Corresponding Author: Asaf Keller, Department of Anatomy & Neurobiology, University of Maryland School of Medicine, 20 Penn Street, Room S216, Baltimore, MD 21201
(410) 706-7307; akeller@som.umaryland.edu

Abstract

Opioid use by pregnant women is an understudied consequence associated with the opioid epidemic, resulting in a rise in the incidence of neonatal opioid withdrawal syndrome (NOWS), and lifelong neurobehavioral deficits that result from perinatal opioid exposure. There are few preclinical models that accurately recapitulate human perinatal drug exposure, and none focus on fentanyl, a potent synthetic opioid that is a leading driver of the opioid epidemic. To more readily investigate the consequences of perinatal opioid exposure, we administered fentanyl to mouse dams in their drinking water throughout gestation and until litters are weaned at postnatal day (PD) 21. First, we found that fentanyl-exposed dams delivered smaller litters, when compared to saccharine-exposed control dams. Twenty-four hours after weaning and drug cessation, fentanyl-exposed mice exhibited signs of somatic withdrawal, and sex-specific weight fluctuations that normalized in adulthood. At adolescence (PD 35) they displayed elevated anxiety-like behaviors and decreased grooming, assayed in the elevated plus maze and sucrose splash tests. Finally, in adulthood (PD 55) they displayed impaired performance in a two-tone auditory discrimination task. Collectively, our findings suggest that we have developed an effective rodent model of NOWS, with high face validity that will allow studying changes associated with perinatal fentanyl exposure across the lifespan.

Keywords: C57BL/6, development, neonatal abstinence syndrome, opiates, postnatal, prenatal

Introduction

The NIH has deemed opioid misuse a national health crisis, with a 30% increase in the rate of opioid overdoses since 2016[74]. The Center for Disease Control and Prevention estimates the total economic burden of opioid misuse in the United States at $78.5 billion annually[14], underscoring the enormous impact opioid misuse has on our health and financial well-being. The overwhelming and rapid nature with which this crisis has materialized is concerning and further warrants a careful understanding of the health consequences of opioid misuse.

Within this growing crisis, individuals between the age of 18 and 25 have exhibited the largest increase in illicit opioid use (Substance Abuse and Mental Health Services Administration, 2013). It is concerning that women of reproductive age show increased incidence of use, given that opioids – both natural and synthetic – can readily cross the placenta and the blood-brain barrier. Indeed, *in utero* opioid exposure is associated with deleterious effects in developing offspring, with more dramatic effects on neurological function observed in infants relative to adults[50,52].

There has been an exponential increase in the distribution of the synthetic opioid, fentanyl, which is now routinely incorporated into the most frequently used narcotics, such as heroin[31,37]. Fentanyl is 50 to 100 times more potent than morphine and is chiefly responsible for the recent increase in synthetic opioid-related overdose deaths[65,66]. The effects that synthetic opioids have on the developmental trajectory of offspring have been incompletely studied, and are needed to develop effective treatment and prevention plans.

Infants that are pre- and peri-natally exposed to opioids exhibit decreased birth weight and body size[3,25,27,28]. Withdrawal symptoms occur in 30-80% of neonates exposed to opioids *in utero*[22], and exhibit disruptions in stress reactivity, altered glucocorticoid levels, hyperactivity, impulsivity, and aggression have been reported later in life[46]. These findings in humans have largely been corroborated and expanded upon by rodent models, which have also revealed several brain

abnormalities associated with opioid exposure[2,15] . Moreover, prenatally exposed rodents have been reported to show abnormalities in motor coordination[6], anxiety[5,68], increased depression-like symptoms[30,39], maze learning and memory impairments[6,34,39,61], as well as altered sexual behavior[70,71], drug sensitivity and analgesic responses[18,63,64] (for review see)[15,52].

Animal models offer distinct benefits for studying the consequences of perinatal opioid exposure. However, previous models have focused on the effects of prenatal morphine exposure, and only during part of the gestational period that is developmentally similar to humans in the second trimester[77], specifically during the rats gestation days 11 to 18[1,32,41,51,59–61,70,71,73]. Others have used continuous exposure to opioids, by way of implantable osmotic minipumps[23,83], a model that does not mimic intermittent use in humans. Few have exposed rodents to prenatal opioids other than morphine[7,7,9,23,24,55,79,80]. Importantly, to our knowledge there exist no preclinical models of perinatal *fentanyl* exposure. Here, we describe a novel model of such exposure by administering fentanyl in the drinking water of pregnant mouse dams. Drug exposure continues in mouse litters until they are weaned at postnatal day (PD) 21. This fentanyl exposure protocol resembles the entire human gestational period[8] and demonstrates its face validity to the human condition, in that it results in postnatal, adolescent and adult phenotypes reminiscent of those in exposed humans.

Materials and methods

Animals. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, as adopted by the NIH, and approved by the Institutional Animal Care and Use Committees at the University of Maryland School of Medicine and at the University of Maryland College Park. Unless otherwise indicated, both male and female C57BL/6J mice were used, and bred in our facility. For auditory discrimination tasks we used the offspring of male CBA/C57BL/6J crossed with female Thy1-GCaMP6f/C57BL/6J mice since CBA strains do not exhibit age-related auditory deficits[17], and readily available from other ongoing calcium imaging studies. When copulatory plugs were identified, we removed the sires, and added fentanyl to the water hydration pouches (see below). Controls received water with 2% saccharin. We replenished the pouches weekly until litters were weaned at postnatal day (PD) 21. We then relocated the offspring and housed them 2 to 5 per cage, in single sex-groups, in a temperature- and humidity-controlled vivarium. Food and water were available *ad libitum,* and lights were maintained on a 12-hour cycle.

Fentanyl citrate. We used 10 µg/mL fentanyl citrate (calculated as free base) in 2% (w/v) saccharin, or 2% saccharin (vehicle control) in the drinking water. This concentration was selected, given that it is the optimal concentration mice will readily self-administer without resulting in motor deficits[17,76], which is well below the mouse oral LD50 of 368 mg/kg (MSDS Cayman Chemical). C57BL/6 mice have an average daily water intake of 4 to 6 mL per day[69], which increases three to four times during pregnancy[42].

Somatic withdrawal behavior. We first habituated mice to the testing room for 1 hour, and scored behavior in real time during a 15 minute observation period, using a modified protocol[36,38]. Withdrawal symptoms were scored as either 1 (present) or 0 (absent) and consisted of 14 distinct behaviors, including unkempt coat, piloerection, persistent trembling, abnormal postures, abnormal gait, wet dog shakes, paw tremors, teeth chattering, genital grooming, excessive eye blinking, ptosis

(orbital tightening), lacrimation, swallowing movements, and diarrhea. We computed the composite score by adding the total score of all withdrawal symptoms.

Elevated plus maze. The elevated plus maze (EPM) is a reliable, canonical test for anxiety-like behavior in rodents[49]. We habituated mice to the testing room for 1 hour and introduced them to the maze by placing them in the central area, facing one of the open arms, for a 5 minute trial. Time spent in the open and closed arms was recorded using computer tracking software (TopScan, CleverSys, Reston, VA).

Splash test. The splash test is a pharmacologically validated behavioral assay demonstrated to parallel other affective-like behavioral assays. We habituated mice to an empty glass cylinder, before spraying their dorsal coat surface 3 times with a 10% sucrose solution. We recorded time spent grooming for 5 minutes.

Two-tone auditory discrimination. We trained mice daily, for seven days, in two 60 minute sessions (one morning, one afternoon). Training consisted of four phases: waterspout habituation, behavioral shaping, single tone training, and two tone operant task. To motivate task acquisition, we water deprived the mice throughout training days and on days when the operant task was conducted. To **habituate** the mice to the water spout, water was made available during 10 second trials, dripping at a slow continuous rate of 15 mL/hr. We randomized inter-trial intervals (ITI) between 30 and 300 seconds and detected licks using the Psibox lickspout[16]. Waterspout habituation lasted for 6 days. We then trained mice to **associate** a tone with licking and water reward delivery. Shaping trials consisted of a 0.5 sec pre-stimulus silence. The target tone was then presented for 1 sec at 0 dB SPL. The response window began at tone onset and ended after a 3 sec post-stimulus silence. A randomized ITI of 5 to 9 s was used between trials. When animals responded at the lick spout during the response window, water was delivered for 2 sec. A conditioning probability was also utilized, where 20% of trials were rewarded with 0.5 sec of water, whether or not the animal responded. To

prevent impulsive licking, the subsequent trial was delayed until mice abstained from licking for 5 sec. Behavioral shaping was done for 4 days. To train in **single tone training** conditions, an early window was introduced for 0.5 sec before the start of each trial; if mice responded during this early window, a timeout of 20 sec was added to the ITI. During this phase, the response window was shortened to 2 sec. The 20% conditioning probability was also removed, making water available only on correct trials. Single tone training lasted for 22 continuous days. The experimental parameters for the **two tone operant task** matched those of single tone training. We tested mice on three distinct target/non-target tone pairs (11000:5000 Hz; 9000:15000 Hz; 7000:13000 Hz) with target to non-target (GO/NOGO) ratios (50/50, 20/80, and 80/20) for 10, 5, and 5 days respectively. We introduced a 20 sec timeout if they responded to the non-target tone during the response window. We assigned mice to respond on a 50/50 target to non-target task to establish basic discrimination differences between groups. We then held the target ratio at 20/80 to determine if discrimination can be increased by making rewarding trials less frequent. Finally, we held the target ratio at 80/20 to determine if frequent target trials would decrease discrimination due to the inability to refrain from responding to infrequent non-target trials (i.e., increased false alarms).

All experimenters were blind to treatment conditions throughout data collection, scoring, and analysis.

Statistical Analyses. Sample size was determined using G*Power software suite (Heinrich-Heine Universität Düsseldorf). Power was set to 0.80 and α at 0.05. We performed statistical tests with Prism 8 (GraphPad). We assumed a sex difference and included sex as a variable. If no statistically significant sex difference or treatment/sex interaction was apparent, animals were grouped and analyzed according to treatment conditions, per NIH recommendations for studying sex differences. We used parametric tests when appropriate assumptions were met, otherwise we used nonparametric tests. Cohen's *d* or η^2 were used for calculating effect size.

Results

Withdrawal Signs

To test the prediction that cessation of perinatal fentanyl exposure induces somatic withdrawal behavior, we compared somatic withdrawal scores between fentanyl exposed and vehicle control mice 24 hours after weaning (PD 22, Fig. 1). We assessed a variety of previously established measures of withdrawal, including unkempt coat, piloerection, persistent trembling, abnormal postures, abnormal gait, wet dog shakes, paw tremors, teeth chattering, genital grooming, excessive eye blinking, ptosis (orbital tightening), lacrimation, swallowing movements, and diarrhea. The somatic withdrawal score is a composite of all withdrawal symptoms that were scored as either a 1 (present) or 0 (absent). Binary scoring was used to avoid score inflation by outlier instances of stereotypy[36,38].

Fentanyl exposed mice (median = 2, 95% CI = 1.79 to 3.15, n = 17) exhibited higher withdrawal scores ($p < 10^{-4}$, Mann-Whitney U = 13.50) compared to vehicle controls (median = 0, 95% CI = -0.03 to 0.43, n = 15), with a large effect size (d = 2.31), suggesting that mice exposed to perinatal fentanyl exhibit withdrawal behaviors 24 hours after weaning.

Litter Size

Dams exposed to fentanyl throughout pregnancy until weaning yielded smaller litter sizes (Fig. 2). Dams exposed to fentanyl (median = 4 pups, 95% CI = 3.93 to 5.34, n = 30) had fewer live pups per litter ($p < 10^{-4}$, t test) relative to vehicle controls (median = 7 pups, 95% CI = 6.22 to 8.39, n = 20), with a large effect size (d = 1.26). These data suggest that dams exposed to fentanyl during pregnancy and throughout gestation have fewer live pups per litter compared to controls.

177 **Animal Weights**

178 To test the prediction that perinatal fentanyl exposure affects body weight across development, we
179 compared the weights at weaning (PD 21), early adolescence (PD 35), and adulthood (PD 55),
180 between fentanyl-exposed and saccharine exposed (vehicle control) mice.

181 Figure 3 depicts the weight, in grams, of mice exposed to fentanyl or vehicle across development. At
182 weaning (Fig. 3A), fentanyl exposed male mice weighed less than controls. There was an interaction
183 between sex and drug exposure ($F_{(1, 44)} = 18.83$, $p < 10^{-4}$), and post hoc comparisons indicate that
184 fentanyl-exposed male mice (median = 7.1, 95% CI = 7.04 to 7.75; $n = 12$) weighed less ($p < 10^{-4,}$
185 Tukey) than vehicle controls (median = 8.7, 95% CI = 8.27 to 9.01, $n = 12$), with a medium effect size
186 ($\eta^2 = 0.09$). There was no difference ($p = 0.99$, Tukey) in weight between fentanyl-exposed female
187 mice (median = 6.4, 95% CI = 6.18 to 6.60, $n = 12$) and vehicle controls (median = 6.4, 95% CI =
188 6.17 to 6.70, $n = 12$).

189 By early adolescence (Fig. 3B), both male and female fentanyl-exposed mice weighed more than
190 their respective sex control. There was no interaction between sex and drug exposure ($F_{(1, 44)} = 1.27$,
191 $p = 0.27$). Tukey's post-hoc multiple comparisons indicate that fentanyl-exposed male mice (median
192 = 19.9, 95% CI = 19.06 to 20.51, $n = 12$) weighed more ($p = 0.03$) than vehicle controls (median =
193 18.2, 95% CI = 16.87 to 19.03, $n = 12$), with a large effect size ($\eta^2 = 0.25$). Fentanyl-exposed female
194 mice (median = 18.1, 95% CI = 16.67 to 18.35, $n = 12$) weighed more ($p = 3^{-4}$) than vehicle controls
195 (median = 14.7, 95% CI = 13.44 to 15.89, $n = 12$), with a large effect size ($\eta^2 = 0.25$).

196 By early adulthood (Fig. 3C), there were no weight differences between fentanyl-exposed mice and
197 controls ($p > 0.05$). These data suggest that male mice exposed to perinatal fentanyl weigh less than
198 vehicle controls at weaning. By adolescence, both fentanyl-exposed male and female mice weigh
199 more than controls. This weight difference is no longer present once these mice reach early
200 adulthood.

Anxiety-like Behavior

Adolescent (6 to 13 year old) children exposed to perinatal opioids exhibit anxiety and aggression[11]. Therefore, we tested the prediction that perinatal fentanyl exposure also influences affective behaviors during adolescence in rodents. We compared the ratio of time fentanyl exposed and control mice spent in open/closed arms of an elevated plus maze (Fig. 4). Male exposed mice displayed more anxiety-like behaviors compared to controls. There was no interaction between sex and treatment ($F_{(1, 62)} = 3.45$, $p = 0.06$), however, there was a sex difference ($F_{(1, 62)} = 10.56$, $p = 0.001$). Tukey's post hoc comparisons indicate that male exposed mice (median = 0.13, 95% CI = 0.09 to 0.21, $n = 9$) had a lower open/closed arm time ratio ($p = 0.01$) than vehicle controls (median = 0.32, 95% CI = 0.25 to 0.56, $n = 12$), with a medium effect size ($\eta^2 = 0.13$). We observed no difference ($p = 0.60$) in the ratio between female exposed mice (median = 0.28, 95% CI = 0.26 to 039, $n = 23$) and vehicle controls (median = 0.34, 95% CI = 0.29 to 0.48, $n = 22$). These data suggest that adolescent male mice exposed to perinatal fentanyl exhibit increased anxiety-like behavior.

We analyzed the time fentanyl exposed and control mice spent grooming themselves during a sucrose splash test (Fig. 5). Female exposed mice spent less time grooming themselves compared to controls. There was no interaction between sex and treatment ($F_{(1, 73)} = 0.19$, $p = 0.66$), however, there was a sex difference ($F_{(1, 73)} = 6.24$, $p = 0.01$). Tukey's post hoc comparisons indicate that female exposed mice (median = 86.81 sec, 95% CI = 84.3 to 112.6, $n = 31$) spent less time grooming themselves ($p = 0.04$) than vehicle controls (median = 118.8 sec, 95% CI = 112.0 to 133.4, $n = 22$), with a small effect size ($\eta^2 = 0.07$). There was no difference ($p = 0.57$) between male exposed mice (median = 71.95 sec, 95% CI = 56.96 to 106.7, $n = 12$) and vehicle controls (median = 107.5 sec, 95% CI = 86.91 to 111.1, $n = 12$). Together, these data suggest that adolescent mice exposed to perinatal fentanyl exhibit aberrant affective behaviors.

Auditory Discrimination

Prenatal exposure to opioids impairs inhibitory control, the voluntary and effortful regulation of avoidance and approach processes, in 2 year old children[33], and is associated with lower performance on perceptual measures of visual, tactile, and auditory tests in 3 to 6 year old children[78]. Here, we tested the prediction that perinatal fentanyl exposure in mice impairs auditory discrimination and inhibitory control.

To test if fentanyl exposure alters sensory perception, we compared performance of exposed and control adult mice on a positive reinforcement auditory discrimination task (Fig. 6). Adult mice were trained and tested on a 50/50 (GO/NOGO) ratio task consisting of a target to non-target tone (see Methods). Next, we examined whether manipulations of the target ratio to non-target ratio differentially impacted tone sensitivity in exposed and control mice. Mice were tested on a target to non-target ratio of 20/80 which we predicted would enhance sensitivity, as well as a target to non-target ratio of 80/20 which we predicted would diminish sensitivity, due to frequent presentation of the target tone which leads to elevated false alarms[72]. We compared the average hits (correctly licked when the target tone was presented), correct rejections (refrained from licking when the non-target tone was presented), total responses (the sum of average hits and false alarms), and d' sensitivity index (a ratio of the hit rate to false alarm rate). Perinatal exposure to fentanyl permanently impaired auditory discrimination and task engagement.

Across weeks 1 (Fig. 6A) and 2 (Fig. 6B) of 50/50 training, fentanyl exposed mice exhibited fewer correct licks when the target tone was presented, compared to vehicle controls. Exposed mice (week 1: median = 0.31, 95% CI = 0.14 to 0.48; week 2: median = 0.40, 95% CI = 0.24 to 0.51; n = 9) had lower average hits (week 1: $F_{(1, 59)} = 914.82$, $p = 0.0004$; week 2: $F_{(1, 59)} = 6.79$, $p = 0.01$) than vehicle controls (week 1: median = 0.66, 95% CI = 0.45 to 0.76; week 2: median = 0.57, 95% CI = 0.45 to 0.68; n = 9), with a large effect size (week 1: $\eta^2 = 0.61$; week 2: $\eta^2 = 0.52$).

Average correct rejections across weeks 1 (Fig. 6C) and 2 (Fig. 6D) were indistinguishable between groups in refraining from licking when the non-target tone was presented. Fentanyl exposed mice (week 1: median = 0.74, 95% CI = 0.59 to 0.85; week 2: median = 0.69, 95% CI = .62 to 0.82; n = 9) had no difference in correct rejections (week 1: $F_{(1, 59)}$ = 3.99, p = 0.05; week 2: $F_{(1, 59)}$ = 0.53, p = 0.47) than vehicle controls (week 1: median = 0.57, 95% CI = 0.48 to 0.71; week 2: median = 0.69, 95% CI = 0.61 to 0.75; n = 9).

On average, total responses across weeks 1 (Fig. 6E) and 2 (Fig. 6F) were lower in fentanyl exposed mice compared to vehicle controls. Exposed mice (week 1: mean = 23.87, 95% CI = 14.89 to 32.85; week 2: mean = 29.83, 95% CI = 23.38 to 36.29; n = 9) made fewer responses (week 1: $F_{(1, 59)}$ = 31.11, $p = 10^{-4}$; week 2: $F(1, 59) = 7.42$, p = 0.009) compared to vehicle controls (week 1: median = 52.83, 95% CI = 44.42 to 56.38; week 2: median = 40.50, 95% CI = 37.63 to 47.63; n = 9), with a large effect size (week 1: η^2 = 0.85; week 2: η^2 = 0.70).

d' sensitivity measures across weeks 1 (Fig. 6G) and 2 (Fig. 6H) were lower in fentanyl exposed mice. Across both weeks 1 and 2, exposed mice (week 1: median = 0.09, 95% CI = -0.10 to 0.31; week 2: median = 0.29, 95% CI = 0.22 to 0.43; n = 9) had decreased sensitivity (week 1: $F_{(1, 59)}$ = 9.5, p = 0.004; week 2: $F_{(1, 59)}$ = 3.73, p = 0.02) relative to vehicle controls (week 1: mean = 50.40, 95% CI = 0.44 to 0.79; week 2: mean = 42.63, 95% CI = 0.69 to 0.96; n = 9), with a large effect size (week 1: η^2 = 0.78; week 2: η^2 = 0.89). No interactions were observed across the two weeks of 50/50 sessions ($p > 0.05$).

We also analyzed d' sensitivity measures across the 20/80 to 80/20 (target/non-target) ratios in mice performing the two-tone operant task (Fig. 6I). d' measures were higher in all mice during 20/80 sessions compared to 80/20 sessions ($F_{(1, 97)}$ = 12.26, p = 0.0007, η^2 = 0.29). This effect was driven by an increase in false alarms (Fig. 6J, $F(1, 97) = 30.07$, $p = 10^{-4}$) but not in hits ($F(1, 97) = 1.82$, p = 0.18). During 20/80 sessions, fentanyl exposed mice made fewer responses compared to controls

($F(1, 59) = 4.84$, $p = 0.03$). We found no difference ($p > 0.05$) between fentanyl exposed and vehicle control mice in d' sensitivity index nor in false alarm rate during 20/80 or 80/20 sessions.

Collectively, this data suggests that when the target ratio was held at 50/50, fentanyl exposed mice made fewer correct responses, fewer overall responses, and exhibited impaired discrimination abilities compared to controls. When we manipulated the frequency of target tones, during 20/80 sessions, exposed mice exhibited fewer responses, however, during 80/20 sessions, it was more difficult for the mice to inhibit licking behavior when the non-target tone was presented. However, no difference between groups was observed in 80/20 sessions. These data suggest that perinatal exposure to fentanyl results in enduring deficits to sensory perception in mice.

Discussion

Opioid Exposure

Animal models of neonatal opioid withdrawal vary greatly in terms of treatment protocols, species, strain, type of opioid, route of administration, drug dose, drug concentrations, and resulting behavioral alteration. Only a small number of studies, including the present study, model opioid exposure in humans throughout pregnancy by exposing pregnant dams throughout gestation and weaning[10,56,57,81]. Almost all previous studies focused on the effects of perinatal exposure to morphine. To our knowledge, ours is the first description of a preclinical model of perinatal *fentanyl* exposure. Fentanyl is more potent, has a faster onset and shorter duration of action, and has a higher abuse potential compared to morphine[40,65]. Although overall overdose deaths might be slightly decreasing, those from fentanyl continue to rise[9]. Therefore, it is important to develop and validate preclinical models of perinatal fentanyl exposure

We chose to administer fentanyl in the drinking water of pregnant dams to better recapitulate the human condition of intermittent opioid use. Exposure to fentanyl through ingestion is not uncommon, as it is responsible for more than half of fentanyl-related overdose deaths[44]. Furthermore, we wanted to avoid chronic stress involved with repeated injections and handling in mice[54], as this might influence behaviors in offspring[62]. We also wanted to avoid continuous administration with pumps and pellets, as these do not mimic intermittent use, and they require surgery. Thus, the intermittent nature of dams self-administering fentanyl better models human scenarios.

Opioid withdrawal in exposed pups

Perhaps the most dramatic and distressing consequence of prenatal opioid exposure in humans is the withdrawal behavior displayed by neonates, collectively referred to as neonatal opioid withdrawal

304 syndrome (NOWS)[12,26,43]. Commonly observed symptoms include irritability, high- pitched crying,
305 tremors, hypertonicity, vomiting, diarrhea, and tachypnea[13].

306 We reasoned that a valid animal model of NOWS should result in a corresponding complement of
307 signs of withdrawal. To our knowledge, few studies assess withdrawal in experimental animals
308 exposed to opioids during the perinatal period[57,67], and none to fentanyl. Here, we demonstrate that
309 perinatal fentanyl exposure results in a large increase in somatic withdrawal scores, consistent with
310 the animals exhibiting a NOWS-like phenotype.

311 **Decreased litter size in exposed dams**

312 Opioid use during pregnancy is associated with premature births, increased risk of spontaneous
313 abortion, and sudden infant death syndrome[75]. Rat dams exposed to morphine before and during
314 pregnancy display hormonal imbalances and irregular estrous cycles that are associated with
315 increased litter morbidity[56,57]. Consistent with these findings, we demonstrate that dams exposed to
316 fentanyl during pregnancy have fewer live pups per litter.

317 **Lower birth weights in exposed pups**

318 Human babies exposed to opioids during pregnancy also have lower birth weights[27,35,75], a finding
319 recapitulated in rats[57] Here we show that perinatal fentanyl exposure is associated with lower
320 weights in males at weaning, and *higher* weights in adolescents of both sexes. By early adulthood
321 weights were similar to those of controls. The transient weight increase in adolescence might be
322 specific to fentanyl exposure, the method of exposure we used, or other, not yet known factors.

323 **Lasting affective deficits in exposed pups**

324 Perinatal exposure to opioids in humans results in complications that continue through to
325 adolescence, including increased risk of developing attention deficit hyperactivity disorder[47],
326 autism[53], autonomic dysregulation[48], and poor performance on standardized high school testing[45].

Children ages 6 to 13 that were exposed to prenatal opioids exhibit affective behavioral deficits[11]. Analogous outcomes are present in rodent models of early opioid exposure with offspring displaying hyperactivity[58], cognitive deficits[7], depressive- and anxiety-like behavior[5,30]. In our model, perinatal fentanyl exposure resulted in aberrant affective behaviors persisting into adolescence, evidenced by their performance on the splash test and the elevated plus maze apparatus.

Lasting sensory deficits in exposed pups

Complications do not end when an exposed baby leaves the intensive care unit. Neonates born with NOWS may display disruptions in the development of somatosensory networks[29], and may develop lasting visual[21], motor[4], and cognitive deficits[20]. Similarly, rats exposed to neonatal morphine at a time equivalent to the third trimester of gestation, have lasting sensory aberrations, including deficits in mechanical and thermal nociception, as well as diminished morphine and stress-induced analgesia[82]. Prenatal morphine exposure altered pyramidal neuron morphology in the visual cortex, specifically small dendritic length, fewer branch numbers, and spine density[41]. Here, we demonstrate that exposed mice have impaired auditory discrimination and lower levels of task engagement in auditory tasks. Surprisingly, perinatal fentanyl exposure did not appear to impact the ability to refrain responding on non-target trials when there was a prepotency to do so.

These deficits might reflect altered frontal-striatal circuits important for task performance and/or abnormal auditory processing. Impaired auditory processing cannot solely account for the observed differences in engagement because our auditory target and non-target stimuli were loud and highly discriminable, and both groups were impacted by altered ratio schedules in that discrimination improved during 20/80 sessions and worsened during 80/20 sessions. Further, in humans, prenatal opioid exposure is *not* associated with hearing impairment, at least in infancy[19,29].

Perinatal fentanyl exposure may also impair basic learning mechanisms: Human toddlers with opioid exposure fail to show trial to trial improvements on the 3-box working memory task[33]. If learning

deficits are at play in our study, they appear to persist even with extended testing (20 sessions); discrimination ability did not differ over days in week 2 during 50/50 testing, and discrimination was still impaired during the last week of testing when ratio manipulations were performed.

Conclusions

We describe a preclinical, rodent model of perinatal fentanyl exposure that recapitulates key aspects of the human condition. This model may allow mechanistic studies of the lasting consequences of perinatal exposure to this potent and widely-used opioid, to enable the development of approaches to prevent or ameliorate these consequences.

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Author contribution

AK, MKL, MRR, POK were responsible for the study concept and design. JBA, ATB, MEF, SST, and CAd conducted the experiments. JBA, ATB, and MEF performed data analysis. JBA, ATB, MRR, and AK drafted the manuscript. DE, NAF, POK, MRR, and AK provided critical revision of the manuscript for content. All authors critically reviewed content and approved the final version for publication.

References

1. Vivolo-Kantor AM, Seth P, Gladden RM, et al. Vital Signs: Trends in Emergency Department Visits for Suspected Opioid Overdoses - United States, July 2016-September 2017. *MMWR Morb Mortal Wkly Rep*. 2018;67(9):279-285. doi:10.15585/mmwr.mm6709e1
2. Florence CS, Zhou C, Luo F, Xu L. The Economic Burden of Prescription Opioid Overdose, Abuse, and Dependence in the United States, 2013. *Med Care*. 2016;54(10):901-906. doi:10.1097/MLR.0000000000000625
3. Gfroerer J. *Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings*. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2013:85-94. https://doi.org/NSDUH Series H-41.
4. Pertschuk LP, Ford DH, Rainford EA. Localization of methadone in fetal rat eye by the immunofluorescence technic. *Exp Eye Res*. 1977;24(6):547-552.
5. Ross EJ, Graham DL, Money KM, Stanwood GD. Developmental consequences of fetal exposure to drugs: what we know and what we still must learn. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol*. 2015;40(1):61-87. doi:10.1038/npp.2014.147
6. Kuczyńska K, Grzonkowski P, Kacprzak Ł, Zawilska JB. Abuse of fentanyl: An emerging problem to face. *Forensic Sci Int*. 2018;289:207-214. doi:10.1016/j.forsciint.2018.05.042
7. Mars SG, Rosenblum D, Ciccarone D. Illicit fentanyls in the opioid street market: desired or imposed? *Addict Abingdon Engl*. December 2018. doi:10.1111/add.14474
8. Stanley TH. The fentanyl story. *J Pain Off J Am Pain Soc*. 2014;15(12):1215-1226. doi:10.1016/j.jpain.2014.08.010
9. Ahmad F, Escobedo L, Rossen L, Spencer M, Warner M, Sutton P. *National Vital Statistics System: Provisional Drug Overdose Death Counts*. Centers for Disease Control and Prevention; 2019. https://www.cdc.gov/nchs/nvss/vsrr/drug-overdose-data.htm. Accessed July 30, 2019.

10. Spencer MR, Warner M, Bastian BA, Trinidad JP, Hedegaard H. Drug Overdose Deaths Involving Fentanyl, 2011-2016. *Natl Vital Stat Rep Cent Dis Control Prev Natl Cent Health Stat Natl Vital Stat Syst*. 2019;68(3):1-19.

11. Kandall SR, Albin S, Lowinson J, Berle B, Eidelman AI, Gartner LM. Differential effects of maternal heroin and methadone use on birthweight. *Pediatrics*. 1976;58(5):681-685.

12. Kandall SR, Doberczak TM, Jantunen M, Stein J. The methadone-maintained pregnancy. *Clin Perinatol*. 1999;26(1):173-183.

13. Binder T, Vavrinková B. Prospective randomised comparative study of the effect of buprenorphine, methadone and heroin on the course of pregnancy, birthweight of newborns, early postpartum adaptation and course of the neonatal abstinence syndrome (NAS) in women followed up in the outpatient department. *Neuro Endocrinol Lett*. 2008;29(1):80-86.

14. Hunt RW, Tzioumi D, Collins E, Jeffery HE. Adverse neurodevelopmental outcome of infants exposed to opiate in-utero. *Early Hum Dev*. 2008;84(1):29-35. doi:10.1016/j.earlhumdev.2007.01.013

15. Holbrook A, Kaltenbach K. Gender and NAS: does sex matter? *Drug Alcohol Depend*. 2010;112(1-2):156-159. doi:10.1016/j.drugalcdep.2010.05.015

16. Ornoy A, Michailevskaya V, Lukashov I, Bar-Hamburger R, Harel S. The developmental outcome of children born to heroin-dependent mothers, raised at home or adopted. *Child Abuse Negl*. 1996;20(5):385-396.

17. Bashore RA, Ketchum JS, Staisch KJ, Barrett CT, Zimmermann EG. Heroin addiction and pregnancy. *West J Med*. 1981;134(6):506-514.

18. Fodor A, Tímár J, Zelena D. Behavioral effects of perinatal opioid exposure. *Life Sci*. 2014;104(1-2):1-8. doi:10.1016/j.lfs.2014.04.006

19. Castellano C, Ammassari-Teule M. Prenatal exposure to morphine in mice: enhanced responsiveness to morphine and stress. *Pharmacol Biochem Behav*. 1984;21(1):103-108. doi: 10.1016/0091-3057(84)90138-2

20. Byrnes EM. Transgenerational consequences of adolescent morphine exposure in female rats: effects on anxiety-like behaviors and morphine sensitization in adult offspring. *Psychopharmacology (Berl)*. 2005;182(4):537-544. doi:10.1007/s00213-005-0122-4

21. Timár J, Sobor M, Király KP, et al. Peri, pre and postnatal morphine exposure: exposure-induced effects and sex differences in the behavioural consequences in rat offspring. *Behav Pharmacol*. 2010;21(1):58-68. doi:10.1097/FBP.0b013e3283359f39

22. McPherson RJ, Gleason C, Mascher-Denen M, Chan M, Kellert B, Juul SE. A new model of neonatal stress which produces lasting neurobehavioral effects in adult rats. *Neonatology*. 2007;92(1):33-41. doi:10.1159/000100084

23. Klausz B, Pintér O, Sobor M, et al. Changes in adaptability following perinatal morphine exposure in juvenile and adult rats. *Eur J Pharmacol*. 2011;654(2):166-172. doi:10.1016/j.ejphar.2010.11.025

24. Slamberová R, Schindler CJ, Pometlová M, Urkuti C, Purow-Sokol JA, Vathy I. Prenatal morphine exposure differentially alters learning and memory in male and female rats. *Physiol Behav*. 2001;73(1-2):93-103. doi:10.1016/s0031-9384(01)00469-3

25. Lu R, Liu X, Long H, Ma L. Effects of prenatal cocaine and heroin exposure on neuronal dendrite morphogenesis and spatial recognition memory in mice. *Neurosci Lett*. 2012;522(2): 128-133. doi:10.1016/j.neulet.2012.06.023

26. Vathy IU, Etgen AM, Rabii J, Barfield RJ. Effects of prenatal exposure to morphine sulfate on reproductive function of female rats. *Pharmacol Biochem Behav*. 1983;19(5):777-780. doi: 10.1016/0091-3057(83)90079-5

27. Vathy IU, Etgen AM, Barfield RJ. Effects of prenatal exposure to morphine on the development of sexual behavior in rats. *Pharmacol Biochem Behav*. 1985;22(2):227-232. doi: 10.1016/0091-3057(85)90382-x

28. Gagin R, Cohen E, Shavit Y. Prenatal exposure to morphine alters analgesic responses and preference for sweet solutions in adult rats. *Pharmacol Biochem Behav*. 1996;55(4):629-634.

29. Sobor M, Timár J, Riba P, et al. Does the effect of morphine challenge change on maternal behaviour of dams chronically treated with morphine during gestation and further on during lactation? *Pharmacol Biochem Behav*. 2010;95(3):367-374. doi:10.1016/j.pbb.2010.02.015

30. Sobor M, Timár J, Riba P, et al. Effects of opioid agonist and antagonist in dams exposed to morphine during the perinatal period. *Brain Res Bull*. 2011;84(1):53-60. doi:10.1016/ j.brainresbull.2010.10.001

31. West JR. Fetal alcohol-induced brain damage and the problem of determining temporal vulnerability: a review. *Alcohol Drug Res*. 1987;7(5-6):423-441.

32. Slamberová R, Rimanóczy A, Cao D, Schindler CJ, Vathy I. Alterations of prenatal morphine exposure in mu-opioid receptor density in hypothalamic nuclei associated with sexual behavior. *Brain Res Bull*. 2005;65(6):479-485. doi:10.1016/j.brainresbull.2005.02.030

33. Slamberová R, Riley MA, Vathy I. Cross-generational effect of prenatal morphine exposure on neurobehavioral development of rat pups. *Physiol Res*. 2005;54(6):655-660.

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

35. Rimanóczy A, Slamberová R, Bar N, Vathy I. Morphine exposure prevents up-regulation of MR and GR binding sites in the brain of adult male and female rats due to prenatal stress. *Int J Dev Neurosci Off J Int Soc Dev Neurosci*. 2006;24(4):241-248. doi:10.1016/j.ijdevneu.2006.04.001

36. Villarreal DM, Derrick B, Vathy I. 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*. 2008;99(3):1235-1242. doi:10.1152/jn.00981.2007

37. Mei B, Niu L, Cao B, Huang D, Zhou Y. Prenatal morphine exposure alters the layer II/III pyramidal neurons morphology in lateral secondary visual cortex of juvenile rats. *Synap N Y N*. 2009;63(12):1154-1161. doi:10.1002/syn.20694

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

39. Hou Y, Tan Y, Belcheva MM, Clark AL, Zahm DS, Coscia CJ. Differential effects of gestational buprenorphine, naloxone, and methadone on mesolimbic mu opioid and ORL1 receptor G protein coupling. *Brain Res Dev Brain Res*. 2004;151(1-2):149-157. doi:10.1016/j.devbrainres.2004.05.002

40. Zissen MH, Zhang G, McKelvy A, Propst JT, Kendig JJ, Sweitzer SM. Tolerance, opioid-induced allodynia and withdrawal associated allodynia in infant and young rats. *Neuroscience*. 2007;144(1):247-262. doi:10.1016/j.neuroscience.2006.08.078

41. Huleihel R, Yanai J. Disruption of the development of cholinergic-induced translocation/ activation of PKC isoforms after prenatal heroin exposure. *Brain Res Bull*. 2006;69(2):174-181. doi:10.1016/j.brainresbull.2005.11.023

42. Schrott LM, Franklin L 'Tonya M, Serrano PA. Prenatal opiate exposure impairs radial arm maze performance and reduces levels of BDNF precursor following training. *Brain Res*. 2008;1198:132-140. doi:10.1016/j.brainres.2008.01.020

43. Chiang Y-C, Hung T-W, Lee CW-S, Yan J-Y, Ho I-K. Enhancement of tolerance development to morphine in rats prenatally exposed to morphine, methadone, and buprenorphine. *J Biomed Sci*. 2010;17:46. doi:10.1186/1423-0127-17-46

490 44. Wu C-C, Hung C-J, Shen C-H, et al. Prenatal buprenorphine exposure decreases neurogenesis
491 in rats. *Toxicol Lett*. 2014;225(1):92-101. doi:10.1016/j.toxlet.2013.12.001
492 45. Wu C-C, Hung C-J, Lin S-Y, et al. Treadmill exercise alleviated prenatal buprenorphine
493 exposure-induced depression in rats. *Neurochem Int*. 2017;110:91-100. doi:10.1016/
494 j.neuint.2017.09.012
495 46. Chen H-H, Chiang Y-C, Yuan ZF, et al. Buprenorphine, methadone, and morphine treatment
496 during pregnancy: behavioral effects on the offspring in rats. *Neuropsychiatr Dis Treat*.
497 2015;11:609-618. doi:10.2147/NDT.S70585
498 47. Devarapalli M, Leonard M, Briyal S, et al. Prenatal Oxycodone Exposure Alters CNS Endothelin
499 Receptor Expression in Neonatal Rats. *Drug Res*. 2016;66(5):246-250. doi:10.1055/
500 s-0035-1569279
501 48. Chen VS, Morrison JP, Southwell MF, Foley JF, Bolon B, Elmore SA. Histology Atlas of the
502 Developing Prenatal and Postnatal Mouse Central Nervous System, with Emphasis on Prenatal
503 Days E7.5 to E18.5. *Toxicol Pathol*. 2017;45(6):705-744. doi:10.1177/0192623317728134
504 49. Frisina RD, Singh A, Bak M, Bozorg S, Seth R, Zhu X. F1 (CBA×C57) mice show superior
505 hearing in old age relative to their parental strains: hybrid vigor or a new animal model for “golden
506 ears”? *Neurobiol Aging*. 2011;32(9):1716-1724. doi:10.1016/j.neurobiolaging.2009.09.009
507 50. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-
508 maze as a measure of anxiety in the rat. *J Neurosci Methods*. 1985;14(3):149-167.
509 51. Surget A, Saxe M, Leman S, et al. Drug-Dependent Requirement of Hippocampal
510 Neurogenesis in a Model of Depression and of Antidepressant Reversal. *Biol Psychiatry*.
511 2008;64(4):293-301. doi:10.1016/j.biopsych.2008.02.022
512 52. Isingrini E, Camus V, Le Guisquet A-M, Pingaud M, Devers S, Belzung C. Association between
513 repeated unpredictable chronic mild stress (UCMS) procedures with a high fat diet: a model of
514 fluoxetine resistance in mice. *PloS One*. 2010;5(4):e10404. doi:10.1371/journal.pone.0010404

53. Francis NA, Kanold PO. Automated Operant Conditioning in the Mouse Home Cage. *Front Neural Circuits*. 2017;11. doi:10.3389/fncir.2017.00010

54. McNally GP, Akil H. Role of corticotropin-releasing hormone in the amygdala and bed nucleus of the stria terminalis in the behavioral, pain modulatory, and endocrine consequences of opiate withdrawal. *Neuroscience*. 2002;112(3):605-617. doi:10.1016/s0306-4522(02)00105-7

55. Luster BR, Cogan ES, Schmidt KT, et al. Inhibitory transmission in the bed nucleus of the stria terminalis in male and female mice following morphine withdrawal. *Addict Biol*. 2019;0(0). doi: 10.1111/adb.12748

56. de Cubas MM, Field T. Children of methadone-dependent women: developmental outcomes. *Am J Orthopsychiatry*. 1993;63(2):266-276.

57. Levine TA, Woodward LJ. Early inhibitory control and working memory abilities of children prenatally exposed to methadone. *Early Hum Dev*. 2018;116:68-75. doi:10.1016/ j.earlhumdev.2017.11.010

58. Wilson GS, McCreary R, Kean J, Baxter JC. The development of preschool children of heroin-addicted mothers: a controlled study. *Pediatrics*. 1979;63(1):135-141.

59. Verde ME, MacMillan NA, Rotello CM. Measures of sensitivity based on a single hit rate and false alarm rate: the accuracy, precision, and robustness of d', Az, and A'. *Percept Psychophys*. 2006;68(4):643-654.

60. Siddiqui A, Haq S, Shaharyar S, Haider SG. Morphine induces reproductive changes in female rats and their male offspring. *Reprod Toxicol*. 1995;9(2):143-151. doi: 10.1016/0890-6238(94)00064-6

61. Siddiqui A, Haq S, Shah BH. Perinatal exposure to morphine disrupts brain norepinephrine, ovarian cyclicity, and sexual receptivity in rats. *Pharmacol Biochem Behav*. 1997;58(1):243-248. doi:10.1016/s0091-3057(97)00012-9

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

63. Wu P-L, Yang Y-N, Suen J-L, Yang Y-CSH, Yang C-H, Yang S-N. 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 Res Int*. 2018;2018:5437092. doi:10.1155/2018/5437092

64. Meert TF, Vermeirsch HA. A preclinical comparison between different opioids: antinociceptive versus adverse effects. *Pharmacol Biochem Behav*. 2005;80(2):309-326. doi:10.1016/ j.pbb.2004.12.002

65. Spencer M Rose, Warner M, Bastian B A, Trinidad J P, Hedegaard H. Drug Overdose Deaths Involving Fentanyl, 2011-2016. *Natl Vital Stat Rep*. 2019;68(3):19.

66. O'Donnell JK, Halpin J, Mattson CL, Goldberger BA, Gladden RM. Deaths Involving Fentanyl, Fentanyl Analogs, and U-47700 - 10 States, July-December 2016. *MMWR Morb Mortal Wkly Rep*. 2017;66(43):1197-1202. doi:10.15585/mmwr.mm6643e1

67. Ryabinin AE, Wang Y-M, Finn DA. Different Levels of Fos Immunoreactivity After Repeated Handling and Injection Stress in Two Inbred Strains of Mice. *Pharmacol Biochem Behav*. 1999;63(1):143-151. doi:10.1016/S0091-3057(98)00239-1

68. Soares-Cunha C, Coimbra B, Borges S, et al. Mild Prenatal Stress Causes Emotional and Brain Structural Modifications in Rats of Both Sexes. *Front Behav Neurosci*. 2018;12:129. doi:10.3389/ fnbeh.2018.00129

69. Devlin LA, Davis JM. A Practical Approach to Neonatal Opiate Withdrawal Syndrome. *Am J Perinatol*. 2018;35(4):324-330. doi:10.1055/s-0037-1608630

70. Jansson LM, Patrick SW. Neonatal Abstinence Syndrome. *Pediatr Clin North Am*. 2019;66(2): 353-367. doi:10.1016/j.pcl.2018.12.006

71. Nellhaus EM, Murray S, Hansen Z, Loudin S, Davies TH. Novel Withdrawal Symptoms of a Neonate Prenatally Exposed to a Fentanyl Analog. *J Pediatr Health Care Off Publ Natl Assoc Pediatr Nurse Assoc Pract*. 2019;33(1):102-106. doi:10.1016/j.pedhc.2018.08.014

72. Finnegan LP, Connaughton JF, Kron RE, Emich JP. Neonatal abstinence syndrome: assessment and management. *Addict Dis*. 1975;2(1-2):141-158.

73. Tao PL, Yeh GC, Su CH, Wu YH. Co-administration of dextromethorphan during pregnancy and throughout lactation significantly decreases the adverse effects associated with chronic morphine administration in rat offspring. *Life Sci*. 2001;69(20):2439-2450. doi:10.1016/s0024-3205(01)01316-9

74. Vucinovic M, Roje D, Vucinovic Z, Capkun V, Bucat M, Banovic I. Maternal and neonatal effects of substance abuse during pregnancy: our ten-year experience. *Yonsei Med J*. 2008;49(5):705-713. doi:10.3349/ymj.2008.49.5.705

75. Ludlow JP, Evans SF, Hulse G. Obstetric and perinatal outcomes in pregnancies associated with illicit substance abuse. *Aust N Z J Obstet Gynaecol*. 2004;44(4):302-306. doi:10.1111/j.1479-828X.2004.00221.x

76. Ornoy A, Segal J, Bar-Hamburger R, Greenbaum C. Developmental outcome of school-age children born to mothers with heroin dependency: importance of environmental factors. *Dev Med Child Neurol*. 2001;43(10):668-675.

77. Rubenstein E, Young JC, Croen LA, et al. Brief Report: Maternal Opioid Prescription from Preconception Through Pregnancy and the Odds of Autism Spectrum Disorder and Autism Features in Children. *J Autism Dev Disord*. 2019;49(1):376-382. doi:10.1007/s10803-018-3721-8

78. Paul JA, Logan BA, Krishnan R, et al. Development of auditory event-related potentials in infants prenatally exposed to methadone. *Dev Psychobiol*. 2014;56(5):1119-1128. doi:10.1002/dev.21160

79. Oei JL, Melhuish E, Uebel H, et al. Neonatal Abstinence Syndrome and High School Performance. *Pediatrics*. 2017;139(2):e20162651. doi:10.1542/peds.2016-2651

80. Sithisarn T, Bada HS, Charnigo RJ, Legan SJ, Randall DC. Effects of perinatal oxycodone exposure on the cardiovascular response to acute stress in male rats at weaning and in young adulthood. *Front Physiol*. 2013;4:85. doi:10.3389/fphys.2013.00085

81. Kivistö K, Nevalainen P, Lauronen L, Tupola S, Pihko E, Kivitie-Kallio S. Somatosensory and auditory processing in opioid-exposed newborns with neonatal abstinence syndrome: a magnetoencephalographic approach. *J Matern-Fetal Neonatal Med Off J Eur Assoc Perinat Med Fed Asia Ocean Perinat Soc Int Soc Perinat Obstet*. 2015;28(17):2015-2019. doi: 10.3109/14767058.2014.978755

82. Gupta M, Mulvihill AO, Lascaratos G, Fleck BW, George ND. Nystagmus and Reduced Visual Acuity Secondary to Drug Exposure In Utero: Long-Term Follow-up. *J Pediatr Ophthalmol Strabismus*. 2012;49(1):58-63. doi:10.3928/01913913-20110308-01

83. Bunikowski R, Grimmer I, Heiser A, Metze B, Schäfer A, Obladen M. Neurodevelopmental outcome after prenatal exposure to opiates. *Eur J Pediatr*. 1998;157(9):724-730.

84. Guo X, Spencer JW, Suess PE, Hickey JE, Better WE, Herning RI. Cognitive brain potential alterations in boys exposed to opiates: In utero and lifestyle comparisons. *Addict Behav*. 1994;19(4):429-441. doi:10.1016/0306-4603(94)90065-5

85. Zhang GH, Sweitzer SM. Neonatal morphine enhances nociception and decreases analgesia in young rats. *Brain Res*. 2008;1199:82-90. doi:10.1016/j.brainres.2007.12.043

86. Grimmer I, Bührer C, Aust G, Obladen M. Hearing in newborn infants of opiate-addicted mothers. *Eur J Pediatr*. 1999;158(8):653-657.

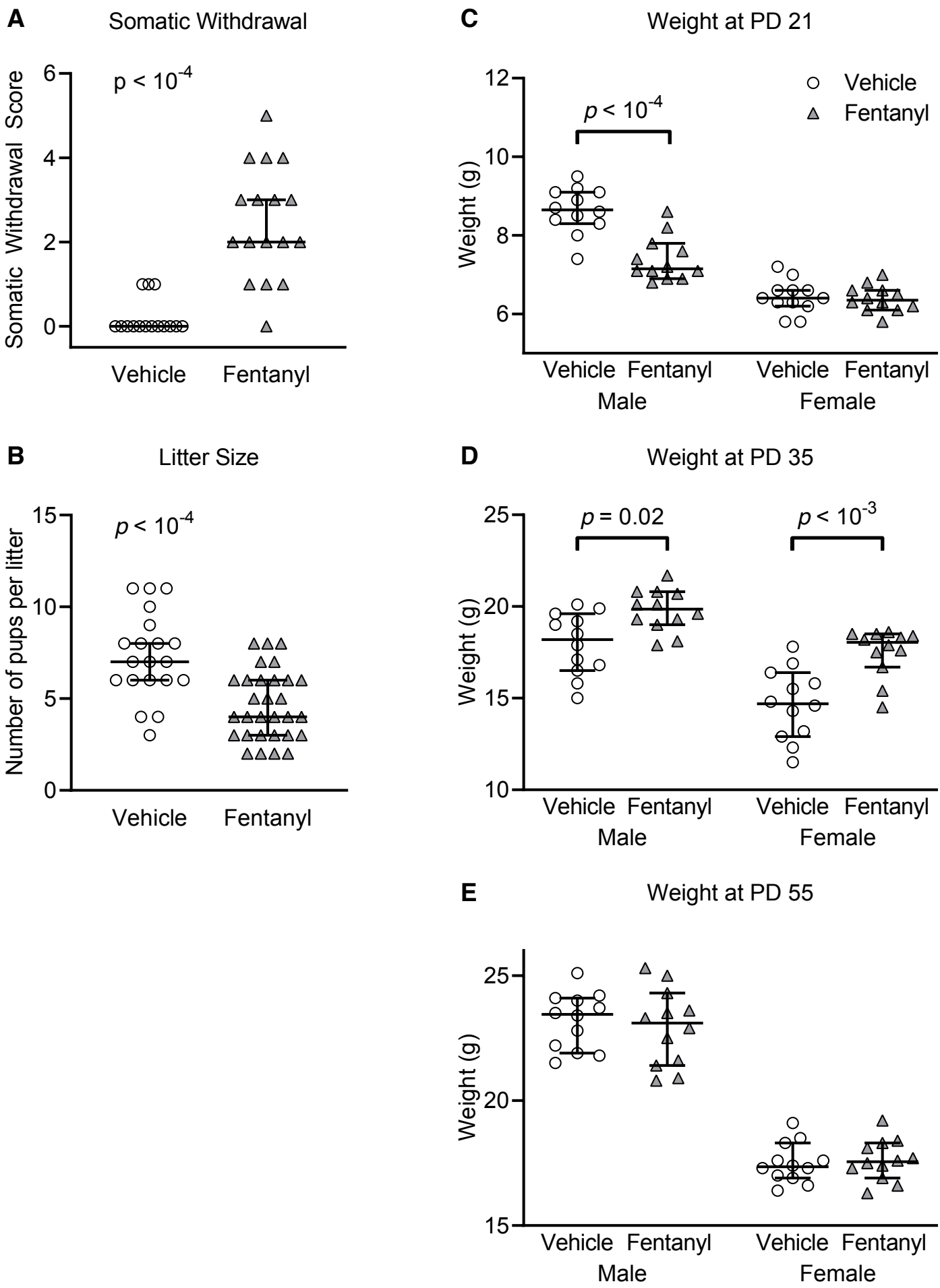
Figure Legends

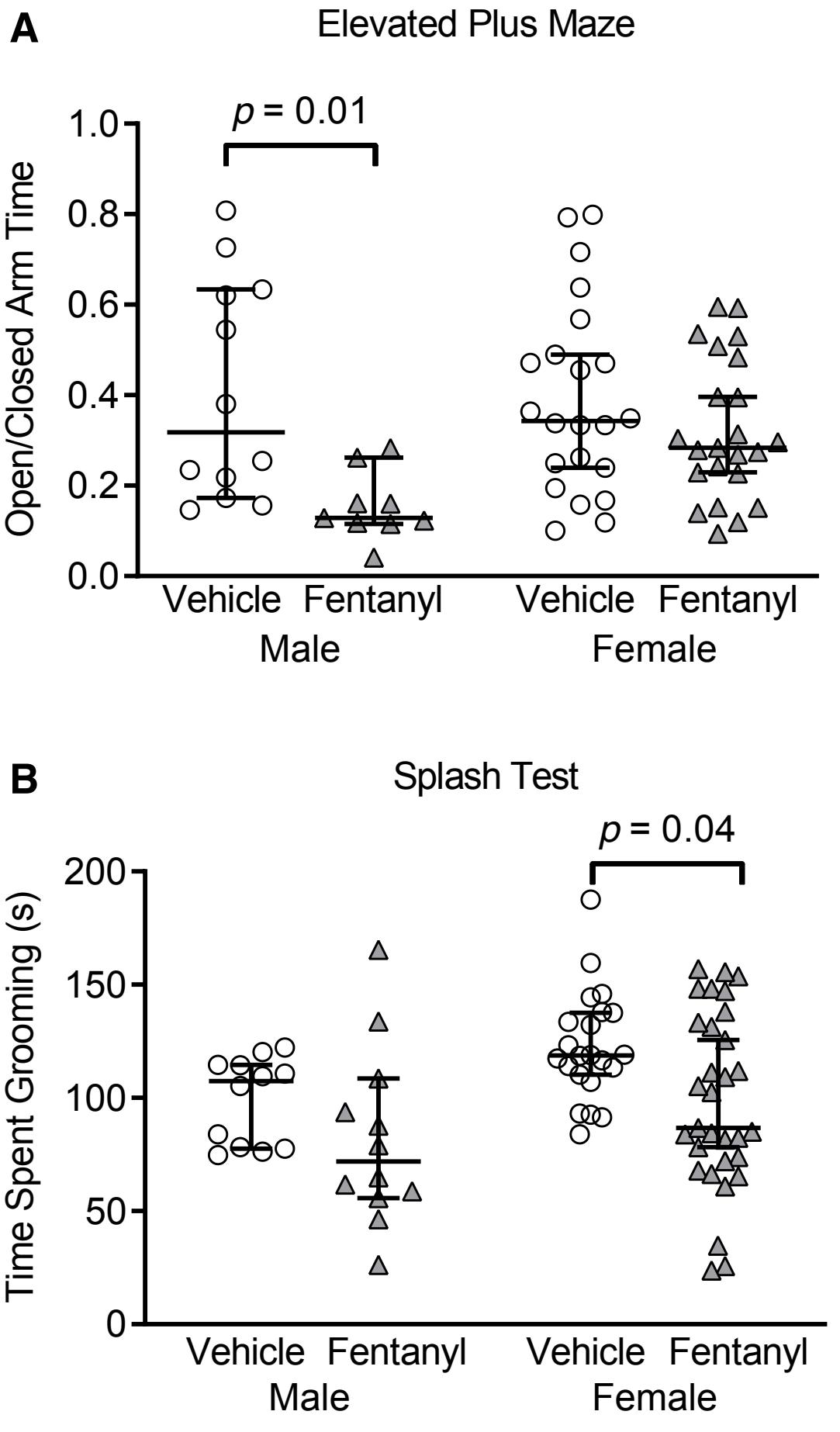
Figure 1. A, Perinatal fentanyl exposure induces somatic withdrawal behavior 24 hours after cessation. Fentanyl exposed mice have higher somatic withdrawal scores, compared with vehicle controls. **B**, Dams exposed to fentanyl during pregnancy have smaller litters, compared to controls. **C - E**, Perinatal fentanyl exposure influences weight across development. **C**, Male exposed mice weigh less than vehicle controls at weaning (PD 21). **D**, By adolescence (PD 35), both male and female exposed mice weigh more than controls. **E**, This weight difference is no longer present once these mice reach early adulthood (PD 55). Data presented are medians with 95% confidence intervals.

Figure 2. A, Perinatal fentanyl exposure significantly increases anxiety-like behavior in adolescent male mice. Male mice exposed to perinatal fentanyl spend less time in open arms of a maze, compared to vehicle controls. **B**, Perinatal fentanyl exposure decreases grooming behavior in adolescent female mice, as assayed with the splash test. Data presented are medians with 95% confidence intervals.

Figure 3. Perinatal fentanyl exposure impairs auditory discrimination and task engagement in adult mice. **A/B**: Fentanyl exposed mice make fewer correct licks when the target tone is presented. **C/D:** There is no difference between groups in refraining from licking when the non-target tone is presented. **E/F:** Exposed mice have fewer responses than vehicle controls. **G/H:** Exposed mice show lower discrimination sensitivity index, compared to vehicle controls. **I:** All mice have significantly higher d' measurements during 20/80 sessions than 80/20 sessions, and there are no differences between treatment groups. **J:** All mice have higher false alarms during 80/20 sessions

than 20/80 sessions, and show no difference between treatment groups. Data presented are medians with 95% confidence intervals.


A
Somatic Withdrawal
Somatic Withdrawal Score
p < 10-4
Vehicle
Fentanyl
B
Litter Size
Number of pups per litter
p < 10-4
Vehicle
Fentanyl
C
Weight at PD 21
Weight (g)
p < 10-4
Vehicle
Fentanyl
Vehicle Fentanyl Vehicle Fentanyl
Male Female
D
Weight at PD 35
Weight (g)
p = 0.02
p < 10-3
Vehicle Fentanyl Vehicle Fentanyl
Male Female
E
Weight at PD 55
Weight (g)
Vehicle Fentanyl Vehicle Fentanyl
Male Female


A
Elevated Plus Maze
p = 0.01
Open/Closed Arm Time
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
Fentanyl
Vehicle
Fentanyl
Male
Female
B
Splash Test
p = 0.04
Time Spent Grooming (s)
200
150
100
50
0
Vehicle
Fentanyl
Vehicle
Fentanyl
Male
Female