Oral Fentanyl Consumption Alters Sleep Rhythms, Promotes Avoidance Behaviors, and Impairs Fear Extinction Learning in Male and Female Mice

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Abstract

The number of opioid overdose deaths has increased over the past several years, mainly driven by an increase in the availability of highly potent synthetic opioids, like fentanyl, in the illicit drug supply. While many previous studies on fentanyl and other opioids have focused on intravenous administration, other routes of administration remain relatively understudied. Here, we used a drinking in the dark (DiD) paradigm to model oral fentanyl self-administration using increasing fentanyl concentrations in male and female mice over 5 weeks. Fentanyl consumption peaked in both female and male mice at the 30 µg/mL dose, with female mice consuming significantly more fentanyl than male mice. Mice consumed enough fentanyl to precipitate withdrawal with naloxone, with males having more severe withdrawal symptoms. Fentanyl consumption disrupted normal sleep rhythms in both male and female mice. We also performed behavioral assays to measure approach/avoidance behavior and reward-seeking during fentanyl abstinence. Female mice showed more avoidance behaviors in the open field assay, whereas male mice showed evidence of these behaviors in the light/dark box assay. Female mice also showed increased reward-seeking in the sucrose preference test. Fentanyl-consuming mice of both sexes showed impaired cued fear extinction learning following fear conditioning. Our experiments show that long-term oral fentanyl consumption disrupts sleep rhythms, promotes anxiety-like behavior, increases motivation for reward, and disrupts fear extinction learning. This model could be useful to further study fentanyl withdrawal syndrome and anxiety-like phenotypes associated with protracted fentanyl withdrawal.

Introduction

Opioid Use Disorder (OUD) has become increasingly prevalent over the last decade with over 26 million people worldwide affected by OUD and 110,360 overdose deaths in March 2022 in the US alone [1,2]. These overdose deaths were primarily attributed to changes in the drug supply and increased availability of highly potent synthetic opioids, such as fentanyl and its analogs [3]. OUD is a chronically relapsing condition characterized by cycles of craving, binge, and withdrawal [4]. In OUD, excessive opioid use occurs to prevent the physical and affective components of withdrawal. Due to recent changes in the drug supply, it is especially important to develop new models to explore withdrawal syndromes associated with fentanyl use to help facilitate the development of new treatments for this disorder.

One aspect of opioid withdrawal syndrome is sleep dysregulation. Sleep disruption commonly occurs during drug consumption and withdrawal from a variety of substances, including alcohol and opioids [5-7]. While studies have observed sleep dysregulation, such as increased time spent in deep sleep, after a single morphine or methadone administration in individuals who do not have history of substance abuse, there is also evidence of increased sleep dysregulation in individuals who have a history with prescription opioid abuse [7,8]. In clinical and animal models, there is evidence of dose-dependent effects of opioids on sleep disruption [9,10]. There have also been observations in animal models of alterations in sleep architecture from exposure to morphine and heroin [11,12]. To our knowledge, the effects of fentanyl on sleep dysregulation are largely unexplored.

In the present study, we trained mice to consume fentanyl orally. While many animal self-administration studies have used intravenous self-administration, it is important to consider that many people who use opioids use other routes of administration. Oral routes of administration are reported as more common in human opioid intake for non-medical purposes, as 97.2% of lifetime non-medical users reported performing oral administration consuming opioids orally at some point during their history of opioid use in a 2005 clinical study [13]. This may be an especially important consideration for fentanyl, where many users self-report a preference for smoking or oral consumption as a route of administration [14,15]. To date there have been a number of animal studies that have demonstrated oral consumption of opioids in rodents using both operant and home-cage drinking [16-25].

Previous studies in our laboratory have utilized a model of precipitated morphine withdrawal to investigate the somatic and negative affective aspects of opioid withdrawal. Notably, we have demonstrated significant sex effects on both neuroplasticity and behavior in response to opioid withdrawal [26,27]. In the present study, we expand on previous studies by developing a single-bottle home-cage drinking-in-the-dark (DiD) model of oral fentanyl self-administration. We investigated fentanyl consumption across a range of concentrations, somatic withdrawal signs following long-term (5 week) fentanyl consumption, behavioral responses in approach/avoidance assays, fear learning and extinction, and sleep dysregulation during fentanyl consumption in both male and female mice.

Methods

Subjects

All Procedures were approved by the University of North Carolina Institutional Animal Use and Care Committee. Male (n=32) and female (n=32) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) at least 8 weeks in age were used in all experiments and singly housed. All animals had food and water *ad libitum* for the duration of the study. After the completion of the study, all animals were euthanized according to IACUC protocols. Mice were only disrupted for weekly cage changes, during the drinking paradigm.

Experimental Design

Experiments were conducted with 3 cohorts of 16 mice each, with each cohort evenly split between water and fentanyl groups and males and females. All animals were 8 weeks old at the start of the experiment. Cohorts 1 and 2 underwent 1 week of baseline sleep measurements in the Piezo-Sleep chambers followed by 5 weeks of fentanyl drinking and sleep measurements in the same sleep chambers. Mice then underwent behavioral testing after 3 days of fentanyl abstinence in the following order, open field, elevated plus maze, light-dark box, sucrose-preference test, and fear conditioning. Cohort 3 was habituated to the reverse light cycle for 1 week and then underwent 5 weeks of fentanyl drinking in standard mouse cages with no sleep measurements. Cohort 3 then underwent behavioral testing in the same order as cohorts 1 and 2.

Drinking in the Dark Model

Mice were randomly assigned to the water or fentanyl group. On baseline days, all mice received drinking water. On the first day of the experiment after baseline, 3 hours into the dark cycle, all mice were given new bottles, with the water group mice receiving water and the fentanyl group mice receiving a solution of fentanyl in drinking water. The

bottles were removed after 4 hours and replaced with drinking water until the next day. Mice received the DiD bottle for 5-days (Monday-Friday) with Saturdays and Sundays off. This cycle was repeated for five weeks, increasing the concentration of fentanyl in drinking water each week (10 ug/mL, 20 ug/mL, 30 ug/mL (for 2 weeks), then 40 ug/mL). It is important to note that the mice were housed in single-bottle cages, and the mice receiving fentanyl did not have access to normal drinking water during the 4-hour drinking sessions. Bottles were filled with drinking water or fentanyl dissolved in drinking water at the beginning of each week and weighed at the beginning and end of the 4-hour drinking session each day to determine the volume consumed for each mouse. One bottle was filled with drinking water and set on an empty cage to account for drip. The amount of fentanyl consumed was calculated as mass of fentanyl consumed divided by weight of the mouse.

Piezo-Sleep System

Mice were single housed in PiezoSleep 2.0 (Signal Solutions, Lexington, KY) 15.5 cm^2 cages in a separate cubicle to minimize disruption from outside noise and light. All mice were maintained on a 12 hour:12 hour reverse light:dark cycle (lights were on 7 PM to 7 AM). PiezoSleep is a non-invasive housing and monitoring system that uses piezoelectric pads beneath the cages to detect the mouse's movement. SleepStats software (SleepStats v4.0, Signal Solutions, Lexington, KY) uses vibrational changes to determine sleeping versus waking respiratory patterns. This method has been validated by visual comparison to electroencephalogram and electromyogram in previous studies [28-30]. Sleep data were evaluated for several parameters, including percent time asleep per hour, percent time asleep per light/dark phase, and mean sleep bout length.

Baseline sleep consisted of at least 5 days of non-disrupted sleep prior to any manipulations.

Open Field

The open field apparatus was a 40 cm x 40 cm clear plastic box with Plexiglass flooring in a sound attenuated chamber with a light source of 55 lux. Mice were placed in the center of open field where locomotor activity was measured based on three zones: surround, center, and corners of the open field. The surround zone excluded the corners. Activity was analyzed with Omnitech Electronics Software (Omnitech Electronics Inc., Columbus, OH).

Elevated Plus-Maze (EPM)

The elevated plus-maze (EPM) apparatus consisted of two open arms and two closed arms, all 77 cm x 77 cm and made of Plexiglass. All arms were connected to a central platform to form the apparatus, which stood 74 cm above the ground. Each mouse was placed on the central platform at the beginning of each trial, and an overhead camera recorded activity for 5 minutes. Time spent in each arm was analyzed with Ethovision software (Noldus, Netherlands).

Light-Dark Box

The Light-Dark box was made of two compartments of equal size. The light compartment was illuminated to 300 lux. Mice were placed in the dark compartment, and an overhead camera recorded activity for 15 minutes. Videos were analyzed with Ethovision software (Noldus, Netherlands).

Sucrose Preference Test (SPT)

Mice were moved to two-bottle choice cages for SPT and allowed to habituate for 5 days. 3 hours into the dark period, all mice were given one bottle of drinking water and one bottle of 1% sucrose in drinking water. The bottles were switched 12 hours into the drinking period and replaced with drinking water after 24 total hours. The same style of bottles were used for both fentanyl DiD and the SPT. All bottles were weighed at the beginning and end of the drinking period. The amount of sucrose consumed was measured by a sucrose preference ratio, dividing the volume of sucrose solution consumed by the total volume of fluid consumed.

Fear Conditioning

Fear learning was conducted over a 4-day protocol On day 1, mice were habituated to the fear conditioning chamber with a shock grid floor (Med Associates, Vermont, USA). On day 2, fear conditioning was performed with a 2 min baseline and 5 tone-shock pairings (tone 30s, 80 db, 3 kHz; shock 0.5 mA, 2 s). Mice then underwent 2 days of fear extinction in a novel context. Following a 2 min baseline, mice received 10 tone presentations (60 s, 80 db, 3 kHz). Behavior hardware was controlled by Ethovision XT and time spent freezing was calculated at baseline and during each tone presentation.

Statistics

All data were analyzed using Graph Pad Prism (version 10.0.02). For most studies males and females were analyzed separately. For the sleep studies, light and dark phase data were analyzed separately. Data are reported as mean $\pm$ SEM. Comparisons were made using student's t-test, regular or repeated measures 2-way ANOVAS, or 3-way ANOVA depending on the number of variables. Drinking data was analyzed using mixed-effect models to account for missing drinking data from bottles leaks. Šídák's

multiple comparisons test or Tukey's multiple comparisons test were used for post-hoc analyses. Significance was defined as $p \leq 0.05$.

Results

Fentanyl drinking

We developed a home-cage fentanyl drinking paradigm modeled on a single bottle DiD method. DiD has long been utilized in alcohol use disorder research as a model of binge alcohol consumption [31]. Due to sole access to alcohol (or another drug) during the active period where there is generally increased consumption, the DiD model promotes higher levels of binge-like drug consumption. In our model we gave mice access to increasing concentrations of fentanyl (10 – 40 µg/mL) dissolved in water over the course of 5 weeks for 4 hours starting 3 hours into the dark cycle. We choose to start at 10 µg/mL fentanyl because previous work has suggested that this concentration is not aversive to rats, although less is known about higher concentrations [32]. Control animals received a bottle of standard drinking water. Mice consumed increasing amounts of fentanyl over each week (**Figure 1A & B**, main effect of dose, $F_{4,102} = 29.08$, $p < 0.0001$). Notably, we observed sex differences with female mice consuming more fentanyl, particularly at the 30 µg/mL concentration where we saw peak consumption (main effect of sex, $F_{1,102} = 24.1$, $p < 0.0001$, Šídák's multiple comparison test, 30 µg/mL, $p = 0.019$, $p = 0.034$). The overall volume of fluid consumed decreased in fentanyl consuming animals suggesting the taste of fentanyl may be aversive at concentrations, particularly at highest concentration tested, 40 µg/mL) (**Figure 1C & D,** time x treatment interaction, $F_{4,184} = 6.86$, $p < 0.0001$; main effect of treatment, $F_{1,46} = 39.52$, $p < 0.0001$). We also assessed changes in body weight over the course of the

drinking paradigm. The body weight of all treatment groups increased over the course of the experiment, (**Figure 1E**, main effect of time, $F_{2.08,9.29} = 70.5$, $p < 0.0001$). However, fentanyl consumption did not alter body weight (main effect of treatment, $F_{1,44} = 0.18$, $p = 0.67$).

Opioid withdrawal behaviors

Previous studies from our lab and others have demonstrated that mice exhibit behavioral symptoms of precipitated opioid withdrawal due to injections of morphine or fentanyl followed by injections of naloxone [26,27]. To assess if mice consumed enough fentanyl to induce withdrawal signs and if there were sex differences in withdrawal symptoms following oral fentanyl consumption, we injected mice with 1 mg/kg naloxone following 4-5 hours of oral consumption of fentanyl (40 µg/mL). We assessed a variety of withdrawal behaviors including fecal boli production, escape jumps, paw tremors, wet dog shakes, abnormal posture, grooming, and jaw tremors. All behaviors were converted to z-scores to account for sex-specific expression of withdrawal behavior and allow for an unbiased assessment of overall withdrawal behaviors between male and female mice. To assess overall withdrawal severity, we averaged the z-scores for all withdrawal symptoms together. The fentanyl drinking group exhibited significantly more withdrawal behaviors than water drinking mice (**Figure 1F**, main effect of treatment, $F_{1,44} = 86.37$. $p < 0.0001$; Tukey's multiple comparisons test, Males $p < 0.0001$, Females $p = 0.0008$). Males exhibited more withdrawal behaviors than females overall (sex x treatment interaction, $F_{1,44} = 11.49$, $p = 0.0015$; main effect of sex, $F_{1,44} = 9.33$, $p = 0.0038$). Male fentanyl drinking mice had significantly greater fecal boli production in withdrawal than female fentanyl mice (**Figure S1A**, sex x treatment effect, $F_{1,44} = 5.23$,

$p = 0.026$, main effect of treatment, $F_{1,44} = 33.27$, $p < 0.0001$, Tukey's multiple comparisons test, $p < 0.0001$). Only males undergoing fentanyl withdrawal had a significant increase in escape jumps (**Figure S1B**, main effect of treatment, $F_{1,44} = 9.64$, $p = 0.003$, Tukey's multiple comparisons test, $p = 0.048$). Both male and female mice undergoing precipitated fentanyl withdrawal had increased paw tremors (**Figure S1C**, main effect of treatment, $F_{1,44} = 108.0$, $p < 0.0001$; Tukey's multiple comparisons test, Males $p < 0.0001$, Females $p < 0.0001$). Both male and female mice undergoing fentanyl withdrawal had significantly increased abnormal postures (**Figure S1D**, main effect of treatment, $F_{1,44} = 104.8$, $p < 0.0001$, Tukey's multiple comparisons, Males $p < 0.0001$, Females $p < 0.0001$) and jaw tremors (**Figure S1G**, main effect of treatment, $F_{1,44} = 169.1$, $p < 0.0001$, Tukey's multiple comparisons test, Males $p < 0.0001$, Females $p < 0.0001$), but there were sex differences with males experiencing more bouts of both abnormal posture (sex x treatment interaction effect, $F_{1,44} = 9.51$, $p = 0.004$) and jaw tremors (sex x treatment interaction, $F_{1,44} = 11.87$, $p = 0.001$) than females. Grooming behaviors were reduced following precipitated fentanyl withdrawal in both male and female mice (**Figure S1F**, main effect of treatment, $F_{1,44} = 7.87$, $p = 0.008$). Male and female mice exhibited differing bouts of wet dog shakes following fentanyl withdrawal with male mice exhibiting more and female mice exhibiting fewer wet dog shakes (**Figure S1E**, sex x treatment interaction effect, $F_{1,44} = 7.62$, $p = 0.008$).

Fentanyl consumption disrupts sleep rhythms in male mice

Previous studies from our lab and others have demonstrated baseline differences in sleep behavior between male and female mice [11]. We also observed baseline sex differences in sleep behavior between male and female mice in this study. Females

slept less than males in the early hours of the light periods but more in the latter hours of the light periods (**Figure S2A**, time x sex effect, $F_{11,330} = 3.488$, $p = 0.0001$). In the dark period, males slept significantly more than females (**Figure S2A**, time x sex effect, $F_{11,330} = 1.860$, $p = 0.0438$). Male mice slept significantly more than female mice, on average, during the dark baseline periods (**Figure S2B**, $t(30) = 3.157$, $p = 0.0036$), but there were no significant differences observed in average sleep during the light baseline periods (**Figure S2B**, $t(30) = 0.5425$, $p = 0.5915$). Female mice primarily had longer baseline sleep bouts in the dark period (**Figure S2C**, time x sex effect, $F_{2,60} = 3.591$, $p = 0.0337$) but showed no difference with male mice in the light period. Accordingly, we chose to analyze all alterations in sleep patterns due to fentanyl consumption separately in male and female mice.

In male mice, we did not see significant changes in time slept across the dark or light period during the first week of 10 µg/mL fentanyl consumption. However, we saw time and treatment interactions for all other doses (**Table S1**). At these doses, the male fentanyl group slept less while the fentanyl bottles were on the cages (Zeitgeber hours 15-19) and slept more immediately after the fentanyl bottles were removed (Zeitgeber hours 20-23) (**Figure 2D, G, J & M**). Male mice slept the same total amount of time during the light period regardless of treatment group, suggesting that mice recovered in the absence of fentanyl (**Figure 2B, E, K, N**). This only differed in light periods of the second week of 30 µg/mL administration, during which the fentanyl group slept more (**Figure 2H**, $t(14) = 2.193$, $p = 0.0457$). During the dark periods for all doses, the fentanyl group had shorter sleep bouts, particularly while fentanyl bottles were present (**Table S2**). At higher doses, the male fentanyl group also had longer sleep bouts in the

hours immediately after fentanyl bottles were removed (**Figure 2C, F, I, L & O**). Sleep bouts were unchanged during the light period. We also assessed changes in sleep bout lengths on days when fentanyl was not available. To do this we subtracted baseline sleep bout length at each time bin from sleep bout length on days when fentanyl was available or on days when fentanyl was not available. We found a greater coherence between baseline changes in sleep bout time for on and off days as fentanyl concentration increased and across time (**Table S5**).

Fentanyl consumption disrupts sleep rhythms in female mice

During the dark periods for all doses, the female fentanyl group showed time and treatment interactions (**Table S3**). Similar to male mice, the female fentanyl group slept less than the female water group while the fentanyl bottles were on the cages (Zeitgeber hours 15-19) and more in the following hours (Zeitgeber hours 20-23), when these bottles were removed (**Figure 3A, D, G, J & M**). The female fentanyl group only slept more during the light periods of the first week of the 30 µg/mL dose (**Figure 3G, Table S3**). There were no differences in total time asleep between treatment groups in either the dark or light periods (**Figure 3B, E, H, K, N**). For sleep bout length, female mice showed time and treatment interactions during fentanyl consumption for all doses except for the first week of the 30 µg/mL fentanyl dose (**Table S4**). The female fentanyl group had shorter sleep bouts while the fentanyl bottles were on cages (**Figure 3C, F, I, L & O**). For the light periods, female mice had similar sleep bout lengths except at the 40 µg/mL fentanyl dose, during which the female fentanyl group had longer sleep bouts (**Figure 3I**, **Table S4**). We also assessed changes in sleep bout lengths on days when fentanyl was not available, using the same baseline subtraction method we used in

males. We found a greater coherence between baseline changes in sleep bout time for on and off days as fentanyl concentration increased and across time (**Table S5**).

Fentanyl consumption alters approach/avoidance behavior

Data from our own lab and other published studies have demonstrated protracted changes in approach/avoidance behavior following opioid use in rodent models [26]. One week after our five-week oral fentanyl administration paradigm, we assessed changes in approach/avoidance behavior using 3 standard behavioral tests, open field (OF), Elevated-plus maze (EPM), and light-dark box (LD). Given the baseline sex differences in fentanyl consumption, we continued to analyze all behavior data separately by sex. There was no significant difference in total distance traveled in the OF assay (**Figure 4A**, main effect of treatment, $F_{1,46} = 0.27$, $p = 0.61$). The female fentanyl group spent a smaller percentage of the total time in the surround than the female water group (**Figure 4B**, main effect of treatment, $F_{1,44} = 5.0$, $p=0.0292$; Šídák's multiple comparisons test, Male $p=0.7901$, Female $p=0.0269$). There were no differences in either males and females on time spent in the center or corners between treatment groups (**Figure 4C-D**, main effect of treatment, center: $p=0.8648$, corners: $p=0.1793$). When examining the OF data across time, we found that fentanyl mice spent less time in the surround than water mice, particularly at later time points (**Figure 4E**, main effect of treatment, $F_{1,46} = 5.01$, $p = 0.03$). There were no differences in time spent in the center across the trial time (**Figure 4F**). However, the fentanyl mice spent significantly more time in the corner of the open field, particularly in the last 10 minutes of the trial (**Figure 4G**, treatment x time interaction, $F_{5,230} = 4.15$, $p = 0.001$, Šídák's multiple comparisons test, $p = 0.036$). In the EPM test, we did not observe any significant differences in open arm entries, time in

open arms, or latency to enter open arms in fentanyl or water controls (**Figure S4A, B, C, & D**). For the LD assay, male mice had a greater latency to enter the light side (**Figure 4H**, main effect of sex, $F_{1,44}$=4.332, p=0.0432), further emphasizing sex differences. However, we did not observe any treatment effects for latency to enter the light side (main effect of treatment, $F_{1,44}$ = 2.77, p=0.1035). The male fentanyl group spent less time on the light side of the box than the male water group, while all female mice spent a similar amount of time on the light side (**Figure 4I**, sex x treatment interaction, $F_{1,44}$ =6.9, p=0.0118; Šídák's multiple comparisons test, Male p=0.0468, Female p=0.3228). We did not observe a main effect of treatment on the number of light side entries (**Figure 4J**, main effect of treatment, $F_{1,44}$ = 0.12, p=0.7307).

Fentanyl consumption alters motivation for reward in female mice

Substance use disorder can result in anhedonia or altered reward signaling [33-35]. To assess changes in anhedonia or motivation for reward, we performed a sucrose preference test. We used a two-bottle choice method with one bottle containing 1% sucrose and the other containing normal drinking water. Only female mice showed a difference in sucrose preference ratio based on treatment. The female fentanyl group had a significantly greater sucrose preference than the female water group (**Figure 4K**, sex x treatment interaction, $F_{1,44}$ =7.115, p=0.0107; main effect of sex, $F_{1,44}$ =15.34, p=0.0003; main effect of treatment, $F_{1,44}$ =6.181, p=0.0168; Šídák's multiple comparisons test, Male p=0.9897, Female p=0.0014).

Fentanyl consumption disrupts fear extinction

Previous studies have demonstrated that chronic opioid consumption alters normal fear learning and extinction [36,37]. To determine if long-term oral fentanyl

consumption disrupts fear learning, mice underwent a fear learning and fear extinction paradigm 1 week into abstinence from fentanyl. On day 1, mice were habituated to the fear conditioning chamber equipped with a shock floor. There were no baseline differences in distance traveled, average velocity, or time spent immobile (freezing) between the treatment groups (data not shown). On day 2, mice underwent fear learning. Mice were presented with 5 tone-shock pairs and time spent freezing was calculated for each pairing. Total time spent freezing increased with each subsequent trial, but there was no significant difference between treatment groups in either male or female mice (**Figure 4L**, main effect of treatment, $F_{1,44} = 0.50$, $p = 0.49$). Mice next underwent fear extinction in a novel context, with a unique cage floor, walls, and scent cues. Mice were presented with 10 tones and freezing time was determined for each tone. On day 1 of extinction, there were no differences in freezing time between the treatment groups (**Figure 4M**, main effect of treatment, $F_{1,44} = 1.37$, $p = 0.25$). However, on day 2 of the extinction trial, both male and female fentanyl drinking mice spent significantly more time freezing relative to the water controls (**Figure 4N**, treatment x tone presentation interaction effect, $F_{10.440} = 3.01$, $p = 0.001$, main effect of treatment, $F_{1,44} = 15.6$, $p = 0.0003$).

Discussion

Oral fentanyl consumption model

Most studies of opioid consumption have focused on intravenous administration, even though other routes of administration, such as vaping or swallowing, are increasingly more common for fentanyl [14,15]. We adapted a DiD model to begin to study the consequences of chronic oral fentanyl consumption. We found that mice consumed

larger amounts of fentanyl across time and with increasing concentrations of fentanyl from 10-40 µg/mL. This was despite an overall decrease in the total volume of fluid consumed with increasing concentrations. Fentanyl consumption stabilized between the 30 and 40 µg/mL concentration which suggests that mice may titrate their consumption to achieve a desired effect, which was previously proposed in an oral oxycodone self-administration paradigm [17]. Alternatively, fentanyl may have an aversive taste at higher concentrations, which reduces consumption at these higher doses. Notably, we never observed any sedative effects of fentanyl in our sleep data, which suggests that consumption is not limited by sedation at higher doses. We observed significant sex differences in fentanyl consumption, with females consuming more fentanyl than males, particularly at the 30 µg/mL concentration. While studies investigating sex differences in oral fentanyl consumption are extremely limited, our work does agree with existing opioid self-administration literature that consistently shows enhanced opioid self-administration in females versus males [21,38-40].

While all mice in the fentanyl group exhibited withdrawal symptoms after naloxone-precipitated withdrawal, we found that male mice showed significantly more withdrawal signs. This is surprising because female mice consumed more fentanyl, which suggests that withdrawal severity is not solely dependent on the amount of fentanyl consumed. Our findings agree with recent work from our laboratory that found that males experienced more severe withdrawal from fentanyl, albeit in a model of non-contingent fentanyl administration [41]. While clinical studies generally report that females experience worse withdrawal symptoms than males, it is important to note that these

studies are typically limited to heroin or illicit use prescription opioid rather than fentanyl [42,43].

Fentanyl consumption disrupts sleep patterns in male and female mice

We found that chronic oral fentanyl administration decreased sleep during the period when fentanyl was available, which is consistent with previous work demonstrating increased locomotor activity after low dose opioid administration [44-46]. It has been reported that opioids have an “inverted U-shape” dose response curve for locomotor stimulation in rodents, where moderate doses produce increased locomotor activity, but high doses induce sedation. Despite the enhanced potency of fentanyl, we did not observe sedative effects of fentanyl at any of the tested concentrations. This may be because of the relatively fast pharmacokinetic clearance of fentanyl prevents high levels of fentanyl from accumulating in the blood or because mice are sensitized to the locomotor stimulating effects of fentanyl over the course of the 5-week paradigm [47,48]. Our data agrees with a previous heroin self-administration study in rats that found that rats receiving heroin slept less than control animals receiving saline during the self-administration session [12]. It is also possible that opioid exposure promotes drug-seeking behaviors that get stronger with long-term exposure. These drug-seeking behaviors may lead to the decrease in sleep as subjects anticipate receiving drug. This may explain why we see greater coherence between sleep-bout patterns on days when fentanyl is present and days without fentanyl exposure during the later weeks of the paradigm. Immediately after fentanyl bottles were removed from cages, we saw an increase in sleep in male and female fentanyl. Coffey et al. did not see this change after their self-administration period of 0.06 mg/infusion of heroin. Instead, in the 2 hours

immediately following self-administration, all drug takers spent the same amount of time awake as the saline group, on average [12]. This may reflect the short half-life of fentanyl relative to heroin [47].

Long-term fentanyl exposure also disrupted normal sleep architecture. Fentanyl mice had shorter sleep-bouts during the period when fentanyl was available and longer sleep bouts during the period immediately after fentanyl access ended. We also observed sex differences whereby fentanyl increased sleep bout length in females during the light period. When acutely administered in healthy adults, opioids have been shown to decrease sleep efficiency and increase awakenings [49]. Long-term opioid consumption is also associated with daytime sleepiness in humans, and we observed a similar effect in our model in the period immediately after fentanyl was removed [50,51]. While we did not differentiate between different stages of sleep in our study, previous work in humans suggests that opioid use may decrease time in periods of deep sleep [8,10,52]. Future studies could investigate changes in REM or non-REM sleep due to fentanyl consumption. Overall, our data support the need for more studies on the consequences of long-term opioid consumption on sleep, and mechanistic studies to examine how long-term opioid consumption alters arousal circuitry in the brain.

Fentanyl consumption alters approach/avoidance behaviors, reward-seeking, and fear extinction.

While the physical symptoms of withdrawal, such as vomiting, diarrhea, and chills, subside shortly after the cessation of opioid consumption, protracted withdrawal symptoms, such as anxiety and depression, can last for months after the cessation of opioid use [53,54]. Our lab has also previously demonstrated protracted withdrawal

symptoms in mice following 6 weeks after naloxone-precipitated morphine withdrawal [26]. We found sex-dependent differences in approach/avoidance behavior after fentanyl administration. Female mice that consumed fentanyl spent less time in the surround area of the open field which suggests more avoidance behavior. All mice in the fentanyl group spent more time in the corner in the last 10 minutes of the open field assay, suggesting impaired habituation. While we did not see any differences in the EPM assay, we found that male mice that consumed fentanyl spent significantly less time in the light-side of the light/dark box and had a trend toward a longer latency to enter the light-side, while females were unchanged. This suggests that fentanyl-consumption may increase anxiety-like behavior, albeit in an assay and sex specific manner. Multiple clinical studies have established a connection between anxiety disorders and opioid use disorder, although the exact causal connection between these disorders is less well understood [55,56]. The association between anxiety and opioid use disorder is especially strong in females [57]. Our data suggests that fentanyl use itself may increase anxiety-like behaviors, although future studies will need to examine more protracted timepoints several weeks after the cessation of opioid use.

Anhedonia is a common feature of opioid use disorder and often serves as a strong predictor of relapse [58,59]. Previous work has suggested that opioid use disrupts normal μ-OR mediated disinhibition of VTA dopaminergic neurons by VTA GABAergic neurons, and this may lead to anhedonia and other negative affective states following opioid use [60]. We assessed anhedonia using the sucrose-preference test and found increased sucrose preference in the female fentanyl-consuming group, but no change in males. This contrasts with previous work on the effects of opioids on anhedonia, and

suggests our fentanyl model enhances motivation for reward, at least in females. This may be due to the fast clearance of fentanyl from the blood relative to morphine, which may differentially alter GABAergic transmission in the VTA [47]. Alternatively, our results may reflect a ceiling effect due to the high preference for the 1% sucrose solution used in our study, and lower concentrations of sucrose may produce different results.

In humans, OUD is associated with mood disorders and PTSD. However, the causal relationship between OUD and PTSD is unclear. Conditioned fear extinction is commonly used to model PTSD in preclinical animal studies and previous work has demonstrated reduced cue-induced extinction learning following chronic morphine exposure and that morphine pretreatment enhanced associative fear learning [36,37]. We found that chronic oral fentanyl exposure did not impact the acquisition of fear learning. However, fentanyl exposure impaired cued extinction learning on the second day of extinction learning, demonstrating that chronic fentanyl consumption disrupts long-term extinction memory. These effects were observed 10 days after the last fentanyl exposure, so they do reflect the effects of acute opioid withdrawal. The basolateral amygdala and pre-frontal cortex (PFC) are two brain regions that are critical for cued fear extinction [61,62]. Notably, μ-OR activation in the PFC disrupts excitatory inputs to the PFC to promote impulsive responses and opioids disrupt normal BLA to mPFC circuitry which may have relevance to fear extinction circuitry [63,64]. While these findings may be important to understanding how fentanyl exposure affects retention of aversive memories, future studies are needed to further investigate molecular mechanisms underlying these changes.

In summary, we developed a mouse model of chronic voluntary home-cage oral fentanyl consumption that results in disruptions to sleep patterns, anxiety-like behaviors, reward motivation, and fear extinction, all of which have been implicated in OUD in humans. Future studies are needed to investigate how fentanyl disrupts key brain circuitry involved in these processes to help develop new treatments for OUD.

Author Contributions

G.K performed experiments, analyzed data, and wrote the manuscript. A.M.D designed and performed experiments, analyzed data, and wrote the manuscript. Z.A.M. designed experiments, wrote the manuscript, and provided funding for the study. All authors approve the final manuscript and agree to be accountable for all aspects for the work.

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Competing Interests

Z.A.M is a co-investigator on SBIR grant 1R43DA057749-01 with Epicypher Inc.

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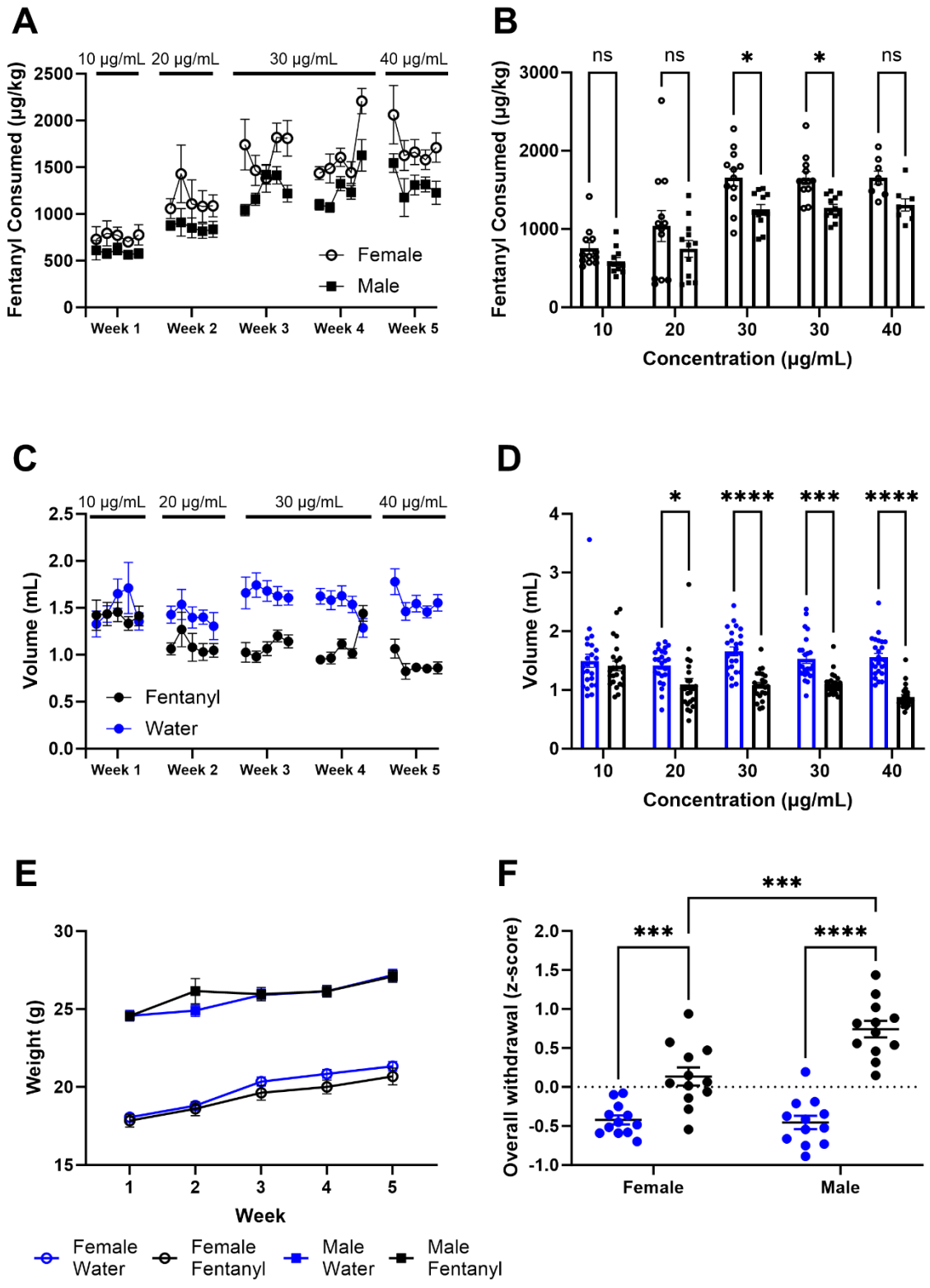
Figures


A
10 µg/mL
20 µg/mL
30 µg/mL
40 µg/mL
Fentanyl Consumed (µg/kg)
Week 1
Week 2
Week 3
Week 4
Week 5
Female
Male
B
ns
ns
*
*
ns
Fentanyl Consumed (µg/kg)
Concentration (µg/mL)
C
Volume (mL)
Fentanyl
Water
D
*
****
***
****
Volume (mL)
Concentration (µg/mL)
E
Weight (g)
Week
Female Water
Female Fentanyl
Male Water
Male Fentanyl
F
***
***
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Overall withdrawal (z-score)
Female
Male

Figure 1. Volume and fentanyl consumed throughout 5-week DiD paradigm and overall withdrawal behavior in female and male mice. **A** Fentanyl consumed (µg/mL) by day in male and female mice. **B** Average fentanyl consumed per week in male and female mice. Females consumed more fentanyl than males, especially at the 30 µg/mL dose. **C** Volume consumed (mL) by day in fentanyl groups and water groups. **D** Average volume consumed per week in fentanyl and water groups. Fentanyl groups consumed significantly less fluid at the 20 µg/mL dose and greater. **E** Weight across 5-week paradigm with no differences observed between treatment groups. **F** Overall withdrawal score (z-score) of fentanyl and water groups by sex. Fentanyl groups exhibited more withdrawal behaviors than water groups in both sexes. Males exhibited more withdrawal behaviors than females. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

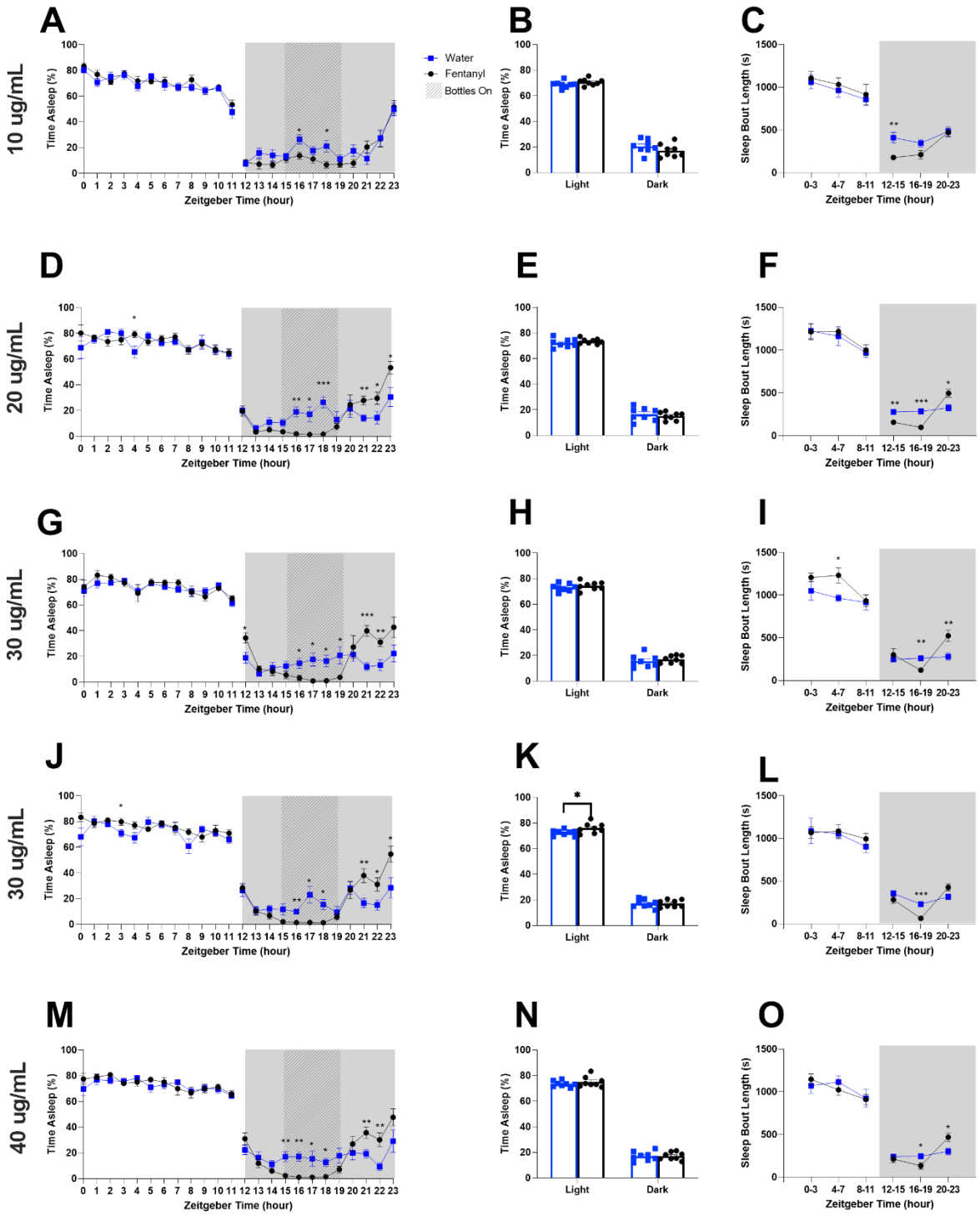

Figure 2. Long-term fentanyl consumption leads to changes in sleep behavior in male mice. **A, D, G, J, M** Time spent asleep by hour in the light (unshaded) and dark (shaded) periods at each dose. Textured region shows the hours at which the fentanyl bottles were placed on cages, during which the fentanyl group slept less than the water group. At the 20 µg/mL dose and greater (**D, G, J, M**), the fentanyl group slept more

than the water group. No differences between treatment groups were observed in the light period. **B, E, H, K, N** Average percent time spent asleep throughout the week at each dose in the light and dark periods. No differences between treatment groups except for at the second 30 µg/mL dose (**K**), where the fentanyl group slept more in the light period. **C, F, I, L, O** Sleep bout length, in seconds) in the light (unshaded) and dark (periods) at each dose. Sleep bout length was binned in 3-hour increments. The fentanyl group revealed shorter sleep bouts during the drinking period at the 20 µg/mL dose and greater (**F, I, L, O**) and longer sleep bouts in the 3-hour period afterward during the weeks of the 20 and 40 µg/mL doses (**F, O**) and the first week of 30 µg/mL dose (**I**). Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

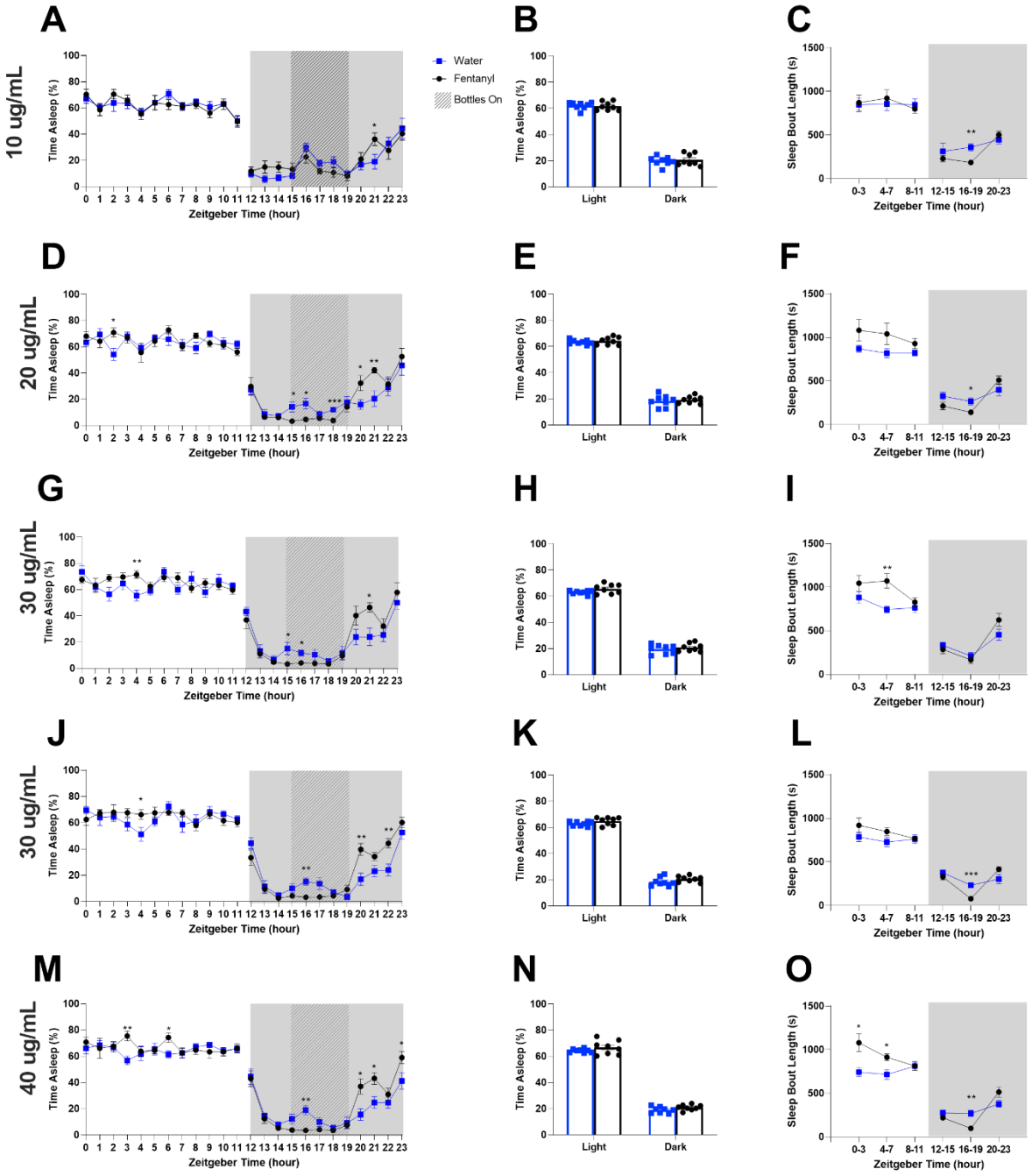

Figure 3. Long-term fentanyl consumption leads to changes in sleep behavior in female mice. **A, D, G, J, M** Time spent asleep by hour in the light (unshaded) and dark (shaded) periods at each dose. Textured region shows the hours at which the fentanyl bottles were placed on cages. The fentanyl group slept less during the drinking period, especially at the 20 µg/mL dose and greater (**D, G, J, M**). **B, E, H, K, N** Average percent time spent asleep throughout the week at each dose in the light and dark periods. No differences between treatment groups. **C, F, I, L, O** Sleep bout length, in seconds in the light (unshaded) and dark (periods) at each dose. Sleep bout length was binned in 3-hour increments. The fentanyl group had shorter sleep bouts during the drinking period

at all doses except for the first week of the 30 µg/mL dose (**I**), during which no differences were observed. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

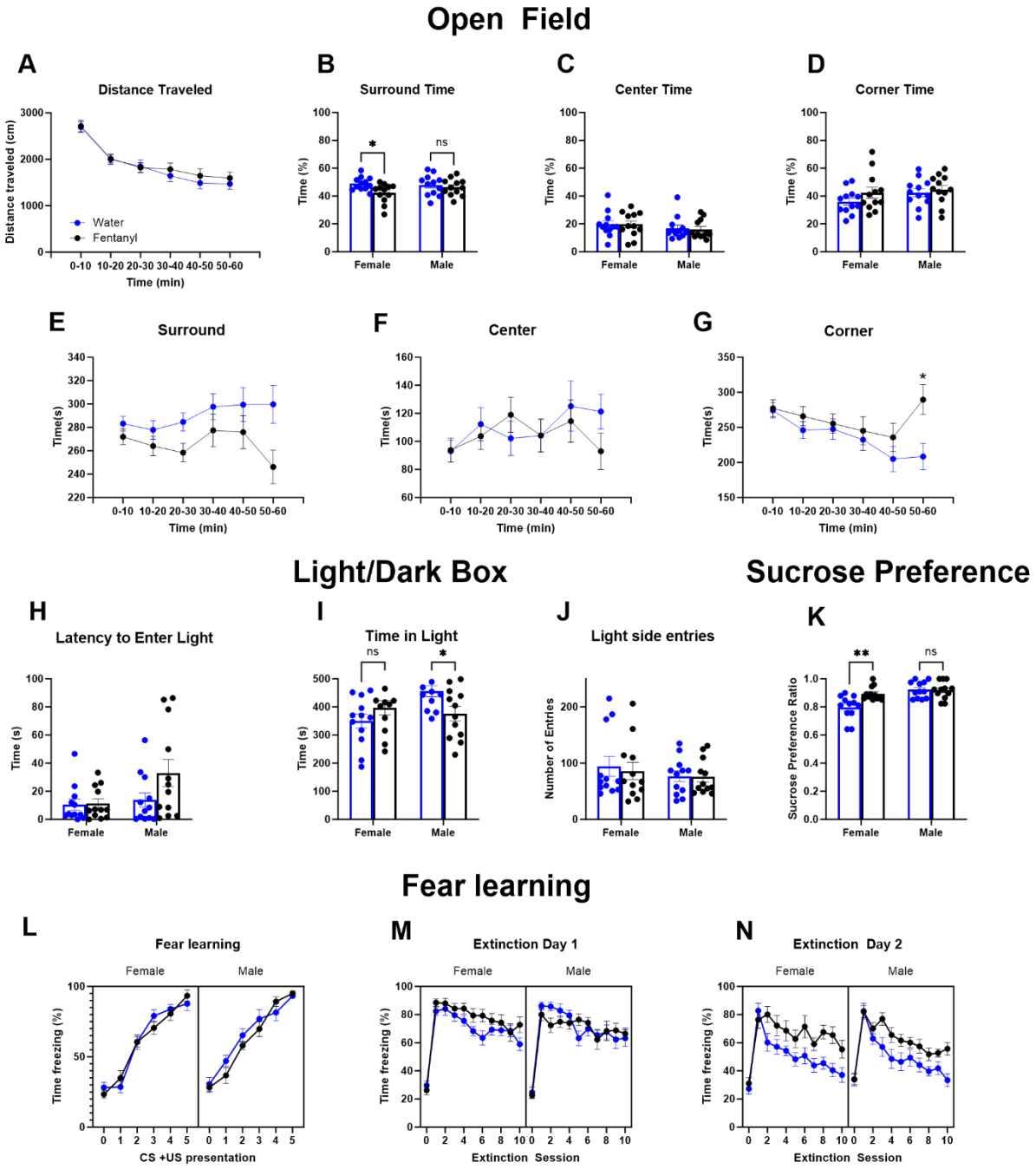

Figure 4. Behavioral assays run one week post DiD paradigm in male and female mice. **A, B, C, D, E, F, G** Show behavior in the open field assay for locomotor activity. **A** Treatment groups showed no differences in total distance traveled when binned in 10-minute increments across the 60-minute assay. **B** Females showed decreased percent time spent in the surround in the fentanyl group, while males showed no differences across treatment groups. **C, D** No differences were observed in the percent time spent in the center or corners between treatment groups for either sex. **E** Fentanyl groups spent relatively less time in the surround region towards the end of the assay. **F**

Treatment groups showed no differences in percent time spent in center at any point throughout the assay. **G** Fentanyl groups spent more time in the corner region towards the end of the assay. **H, I, J** Show behavior in the light/dark box assay. **H** There were no significant differences in the latency, in seconds, to enter the light side. **I** The male fentanyl group spent less time in the light overall than the male water group, while females did not show significant differences. **J** For both sexes, there were no differences in the amount of times subjects entered the light side. **K** Shows the sucrose preference test with 1% sucrose solution. The female fentanyl group showed a higher ratio of sucrose solution to total fluid consumed than the female water group, while males showed no differences. **L, M, N** Show the fear conditioning assay. **L** Percent time spent freezing during fear learning was consistent across treatment groups for both sexes. On extinction days, cues were played every two minutes for one minute. **M** On the first day, there was no significance in freezing between each group. **N** On the second day, the fentanyl groups for both sexes showed significantly more time freezing across all cues. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Supplemental Materials

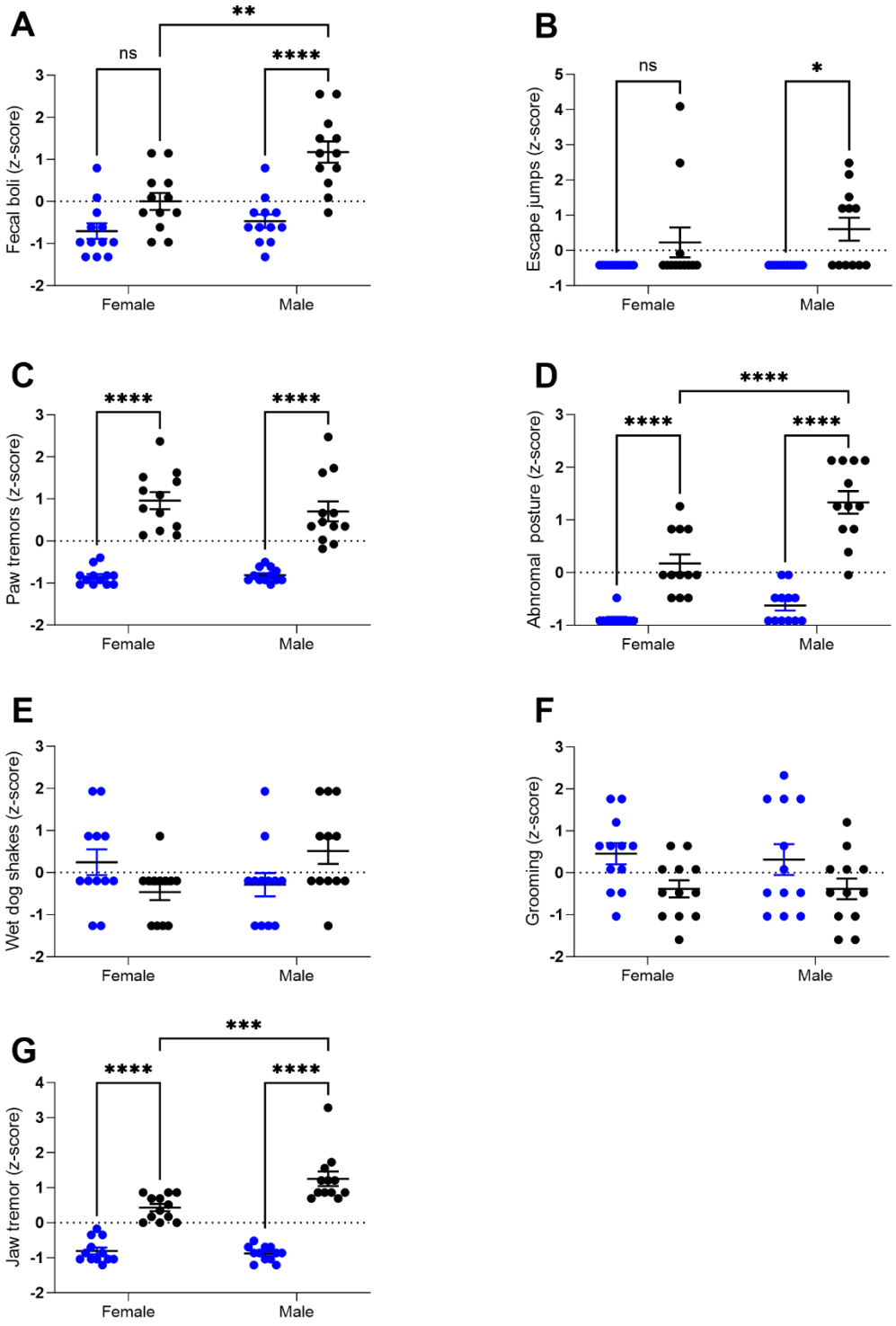

Figure S1. Long-term fentanyl consumption shows sex differences in withdrawal behaviors. **A** Male fentanyl group produced more fecal boli than both male water group and female fentanyl group. **B** Only males exhibited more escape jumps in the fentanyl

group. Females showed no differences between treatment groups. **C** Both male and female fentanyl groups had increased paw tremors than water groups. **D** Both male and female fentanyl groups had more instances of abnormal posture than water groups, but male fentanyl group had more instances than female fentanyl group. **E,F** No differences were observed for wet dog shakes and grooming. **G** Both male and female fentanyl groups had increased jaw tremors, but male fentanyl group revealed more jaw tremors than female fentanyl group. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

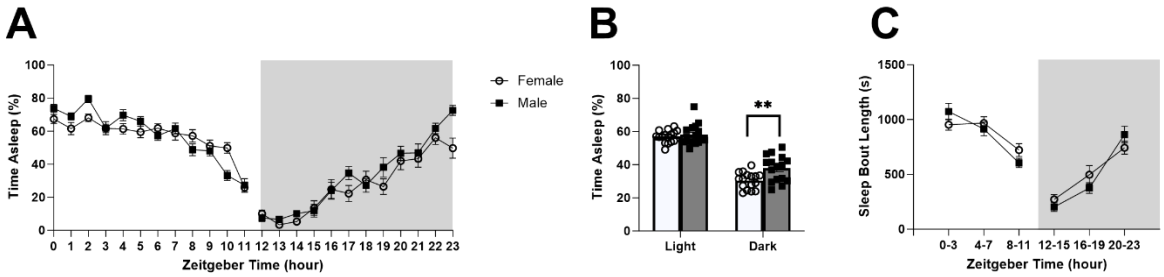

Figure S2. Female and male mice show differences in baseline sleep. **A** Females slept less in the earlier hours and more in the latter hours of the light periods. Females also slept less in the dark periods, especially in the latter hours. **B** Females slept less, on average, during the dark periods of baseline days. No sex differences were observed during the light periods. **C** Females generally had longer sleep bouts during the dark periods. No sex differences were observed during the light periods. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

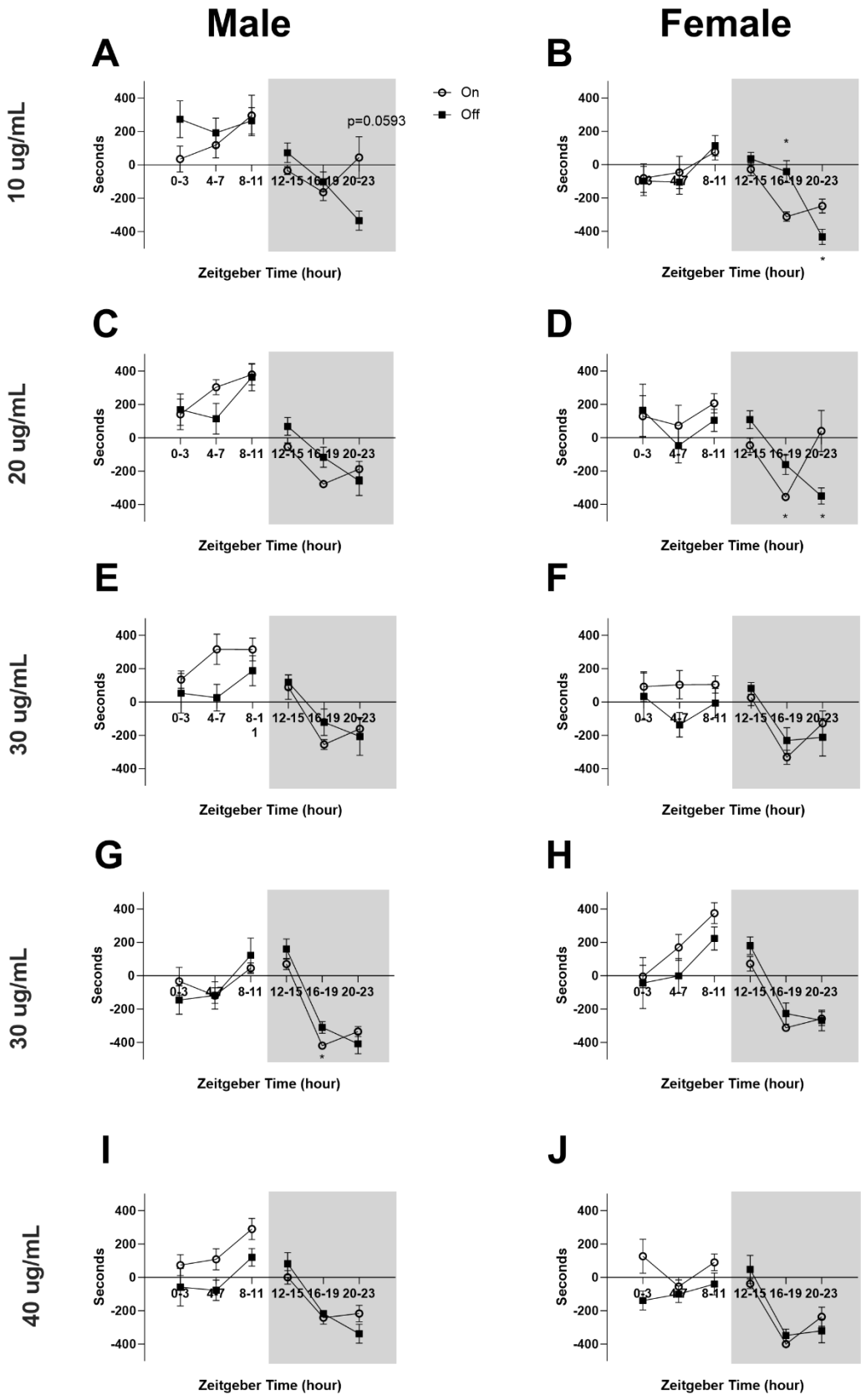
Male
Female
A
B
C
D
E
F
G
H
I
J
10 ug/mL
20 ug/mL
30 ug/mL
30 ug/mL
40 ug/mL
On
Off
p=0.0593
Seconds
400
200
0
-200
-400
0-3
4-7
8-11
12-15
16-19
20-23
Zeitgeber Time (hour)

Figure S3. Sleep bout lengths on days with and without bottles become more similar with increasing dose and exposure to fentanyl. **A,B** At the 10 µg/mL dose, male and the female fentanyl group showed significant differences in sleep bout lengths on the on and off days, especially in the latter hours of the dark period. **C** At the 20 µg/mL dose, male fentanyl groups started to show similar sleep bout lengths in both the light and dark periods of the on and off periods. **D** While the female fentanyl group still showed similar sleep bout lengths in the light period of the on and off days, they still differed in the dark periods at the 20 µg/mL dose. **E,F** During the first week of the 30 µg/mL dose, male and female fentanyl groups showed similar sleep bout lengths in the light and dark periods of the on and off days. **G,H** During the second week of the 30 µg/mL dose, male and female fentanyl groups showed stronger evidence of similar sleep bout lengths in both the light and dark periods of the on and off days. **I,J** At the 40 µg/mL dose, male and female fentanyl groups stayed consistent in similar sleep bout lengths in the light and dark periods on the on and off days. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

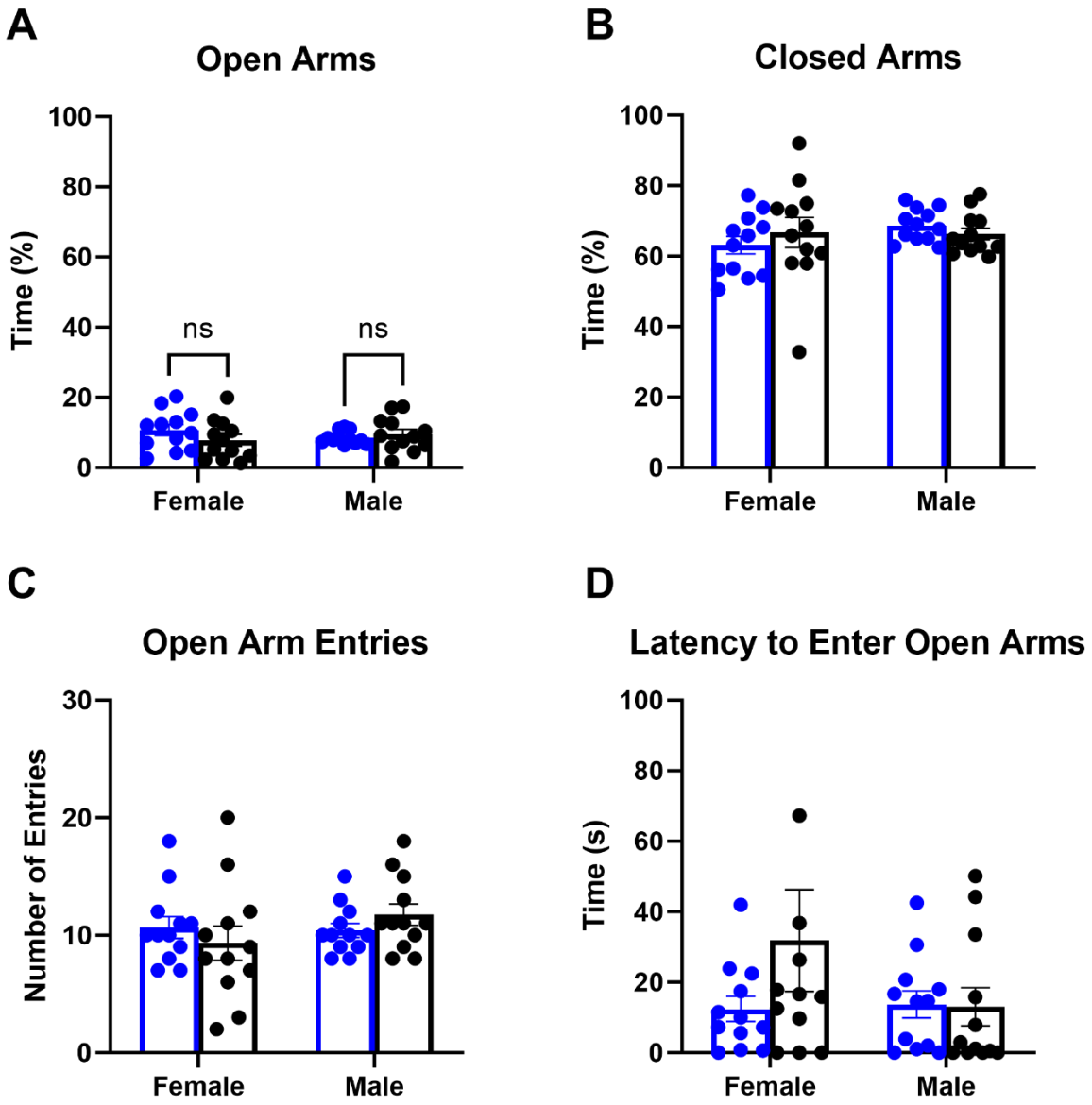

Figure S4. Male and female mice did not show behavioral differences in approach-avoidance conflict through the elevated plus maze assay. **A,B** Both treatment groups of male and female mice did not show difference in the time spent in either the open or closed arms of the maze. **C** Male and female mice had about the same number of entries into the open arms, regardless of treatment. **D** The female fentanyl group showed some evidence of a longer latency to enter the open arms of the maze, but this was not a significant difference from the female water group. Male fentanyl and water groups did not show differences in the latency to enter the open arms. Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Table S1. Two-Way ANOVAs for Male Mice Hourly Sleep Percent

			Main Effect of Treatment		Interaction Effect (Time x Treatment)	
Week	**Dose (µg/mL)**	**Period**	**$F_{1,14}$**	**p**	**$F_{11,154}$**	**p**
1	10	Light	2.036	0.1755	0.6818	0.7543
		Dark	3.768	0.0726	1.607	0.1016
2	20	Light	0.9448	0.3475	1.335	0.2099
		Dark	0.8930	0.3607	5.884	<0.0001
3	30	Light	0.7316	0.4068	0.5417	0.8724
		Dark	0.7513	0.4007	6.497	<0.0001
4		Light	5.007	0.0420	1.715	0.0747
		Dark	0.006816	0.9354	5.586	<0.0001
5	40	Light	0.8436	0.3739	0.6769	0.7589
		Dark	0.0594	0.8109	5.211	<0.0001

Table S2. Two-Way ANOVAs for Male Mice Sleep Bouts

			Main Effect of Treatment		Interaction Effect (Time x Treatment)	
Week	**Dose (ug/mL)**	**Period**	**$F_{1,14}$**	**p**	**$F_{2,28}$**	**p**
1	10	Light	0.3050	0.5895	0.02200	0.9783
		Dark	5.992	0.0282	3.795	0.0348
2	20	Light	0.08003	0.7814	0.1506	0.8609
		Dark	4.213	0.0593	15.37	<0.0001
3	30	Light	3.370	0.0877	1.719	0.1976
		Dark	2.182	0.1617	7.141	0.0031
4		Light	0.1084	0.7468	0.4753	0.6266
		Dark	2.477	0.1379	8.903	0.0010
5	40	Light	0.01292	0.9111	1.195	0.3176
		Dark	0.1876	0.6715	5.199	0.0120

Table S3. Two-Way ANOVAs for Female Hourly Sleep Percent

			Main Effect of Treatment		Interaction Effect (Time x Treatment)	
Week	**Dose (µg/mL)**	**Period**	$F_{1,14}$	**p**	$F_{11,154}$	**p**
1	10	Light	0.1148	0.7398	0.4509	0.9300
		Dark	0.1730	0.6838	1.940	0.0383
2	20	Light	0.4573	0.5099	1.684	0.0818
		Dark	0.1555	0.6993	3.097	0.0009
3	30	Light	3.347	0.0887	1.971	0.0348
		Dark	0.3716	0.5519	2.631	0.0042
4		Light	2.391	0.1443	1.387	0.1842
		Dark	1.794	0.2018	5.649	<0.0001
5	40	Light	1.372	0.2611	1.675	0.0839
		Dark	3.257	0.0927	4.274	<0.0001

Table S4. Two-Way ANOVAs for Female Mice Sleep Bouts

			Main Effect of Treatment		Interaction Effect (Time x Treatment)	
Week	**Dose (µg/mL)**	**Period**	$F_{1,14}$	**p**	$F_{2,28}$	**p**
1	10	Light	0.02403	0.8790	0.4620	0.6347
		Dark	1.322	0.2695	3.677	0.0382
2	20	Light	3.545	0.0807	0.6859	0.5119
		Dark	1.669	0.2173	3.787	0.0350
3	30	Light	6.997	0.0192	2.964	0.0680
		Dark	0.4747	0.5021	2.475	0.1024
4		Light	1.833	0.1972	1.469	0.2473
		Dark	1.089	0.3143	7.978	0.0018
5	40	Light	5.490	0.0344	10.65	0.0004
		Dark	0.8624	0.3688	8.823	0.0011

Table S5. Two-Way ANOVAs for Difference in Sleep Bout Lengths While Bottles are On or Off and Baseline Values

				Main Effect of Treatment		Interaction Effect (Time x Treatment)	
Week	**Dose (µg/mL**	**Period**	**Sex**	**$F_{1,14}$**	**p**	**$F_{2,28}$**	**p**
1	10	Light	Male	0.6843	0.4220	2.482	0.1018
			Female	0.02382	0.8796	0.3049	0.7396
		Dark	Male	1.484	0.2433	7.576	0.0023
			Female	1.280	0.2769	16.85	<0.0001
2	20	Light	Male	0.6264	0.4419	1.198	0.3167
			Female	0.2311	0.6381	0.6310	0.5394
		Dark	Male	2.429	0.1414	2.779	0.0793
			Female	0.05601	0.8163	13.70	<0.0001
3	30	Light	Male	4.105	0.0622	1.003	0.3795
			Female	2.017	0.1775	0.7582	0.4779
		Dark	Male	0.3103	0.5863	0.9483	0.3995
			Female	0.1392	0.7147	1.070	0.3566
4		Light	Male	1.363	0.2625	0.4682	0.6309
			Female	0.01586	0.9016	1.690	0.2028
		Dark	Male	2.624	0.1275	0.7828	0.4669
			Female	1.841	0.1963	2.656	0.0879
5	40	Light	Male	3.737	0.0737	0.1503	0.8612
			Female	3.742	0.0735	3.360	0.0492
		Dark	Male	0.03744	0.8493	1.905	0.1676
			Female	0.2020	0.6600	1.217	0.3112