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

Anthony M. Downs, Ph.D.[a,b*], Gracianne Kmiec [a*], & Zoé A. McElligott Ph.D.[a,b,c]

[a] Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599
[b] Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599
[c] Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599
[*]Authors contributed equally to the manuscript

Corresponding authors: Zoe A. McElligott Ph.D.**:** zoemce@email.unc.edu
Anthony M. Downs Ph.D.: anthony_downs@med.unc.edu

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 sufficient fentanyl such that withdrawal was precipitated with naloxone, with males having more severe withdrawal symptoms, despite lower pharmacological exposure. Fentanyl consumption disrupted normal sleep rhythms in both male and female mice. We also performed behavioral assays to measure avoidance behavior and reward-seeking during fentanyl abstinence. Female mice displayed 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 exhibited increased reward-seeking in the sucrose preference test. Fentanyl-consuming mice of both sexes showed impaired cued fear extinction learning following fear conditioning and increased excitatory synaptic drive and increased excitability of BLA principal neurons. Our experiments demonstrate that long-term oral fentanyl consumption results in wide-ranging physiological and behavioral disruptions. This model could be useful to further study fentanyl withdrawal syndrome, fentanyl seeking, and behaviors 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.

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 increasingly more common with opioids, especially early in the history of illicit use [5]. 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 [6,7]. 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 [8-17].

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 [18-20]. Studies from both our lab and in clinical populations have also demonstrated disruptions in sleep quality and sleep architecture following both acute and chronic opioid administration [21-27]. 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, behavioral responses in approach/avoidance assays, fear learning and extinction, basolateral amygdala (BLA) physiology, 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=24) and female (n=24) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) at 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.

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. Cohorts 1 and 2 underwent 1 week of baseline sleep measurements in 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. Following fear conditioning, all cohorts were returned to standard mouse cages and resumed the fentanyl DiD paradigm with 30 µg/mL fentanyl for at least 1 week. Animals were then sacrificed for electrophysiological recordings 3-5 days into abstinence from fentanyl.

Drinking in the Dark (DiD) Model

On the first day of the drinking paradigm, mice were given either a bottle of plain water or fentanyl dissolved in drinking water beginning 3 hours into the dark cycle. The bottles were removed after 4 hours and replaced with drinking water until the next day. Mice underwent DiD for 5 days (Monday-Friday) with Saturdays and Sundays off. This cycle was repeated for 5 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). 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 weighed at the beginning and end of each drinking session to determine the amount consumed. One bottle was filled with drinking water and set on an empty cage to account for drip.

Piezo-Sleep System

Sleep was assessed using the Piezo Sleep System as previously described [18,28-30], additional details are in the Supplement.

Opioid withdrawal behaviors

Opioid withdrawal behaviors were assessed as previously described [18,19,31], see Supplement for full details.

Avoidance assays

Open field, elevated plus-maze, and light-dark box were performed as previously described [32], see Supplement for full details.

Sucrose Preference Test (SPT)

SPT was performed according to published methods [33], see Supplement for full details.

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.

Brain slice preparation

Brain slices were prepared for whole-cell electrophysiology as previously described [34]. See supplement for more details.

Whole-cell recordings

Recordings were conducted as previously described [35,36], see Supplement for full details. Cells in which access resistance changed greater than 20% were excluded from analysis. 1-3 cells were recorded from each animal for each set of experiments. BLA

principal neurons were identified by their anatomical location, low membrane resistance < 50 MΩ, and high capacitance > 100 pF.

Drugs

Fentanyl citrate was obtained from Spectrum Pharmacy Products (New Brunswick, NJ, USA). Naloxone hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Statistics

All data were analyzed using Graph Pad Prism (version 10.0.02). For most studies males and females were analyzed separately due to significant differences in fentanyl consumption. For the sleep studies, light and dark phase data were analyzed separately as in previous studies from our lab [18,37]. Data are reported as mean ± 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. 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. 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. We chose 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 [8,38,39]. Control animals received a bottle of standard drinking water. Mice consumed increasing amounts of fentanyl over each week (**Figure 1A & B**). Notably, we observed sex

differences with female mice consuming more fentanyl, particularly at the 30 µg/mL concentration where we saw peak consumption. The overall volume of fluid consumed decreased in fentanyl consuming animals suggesting the taste of fentanyl may be slightly aversive at higher concentrations, particularly at highest concentration tested, 40 µg/mL (**Figure 1C & D**). 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 and was unaltered by fentanyl consumption (**Figure 1E**).

Opioid withdrawal behaviors

To assess if mice consumed sufficient 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 [31]. 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**). Males exhibited more withdrawal behaviors than females overall. See Supplement for an analysis of individual withdrawal behaviors.

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 [18,40]. We also observed baseline sex

differences in sleep behavior between male and female mice in this study (See Figure S2 & Supplemental Results). 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 sleep 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**). 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 avoidance behaviors

Data from our own lab and other published studies have demonstrated protracted changes in avoidance behavior following opioid use in rodent models [19]. One week after our five-week oral fentanyl administration paradigm, we assessed changes in 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 were no significant

differences in total distance traveled in the OF assay (**Figure 4A**). The female fentanyl group spent a smaller percentage of the total time in the surround than the female water group (**Figure 4B**). There were no differences between treatment groups in either males and females on time spent in the center or corners (**Figure 4C-D**). 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**). 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**). 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, we did not observe any treatment effects for latency to enter the light side (**Figure 4H)**. 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**). We did not observe treatment effects on the number of light side entries (**Figure 4J**).

Fentanyl consumption alters motivation for reward in female mice

Substance use disorder can result in anhedonia or altered reward signaling [41-43]. To assess changes in anhedonia or motivation for reward, we performed a sucrose preference test. Only female mice showed a difference in sucrose preference ratio based on treatment. Surprisingly, the female fentanyl group had a significantly greater sucrose preference than the female water group (**Figure 4K**).

Fentanyl consumption disrupts fear extinction

Previous studies have demonstrated that chronic opioid consumption alters normal fear learning and extinction [44,45]. 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. Total time spent freezing increased with each subsequent trial, but there were no significant differences between treatment groups in either male or female mice (**Figure 4L**). Mice next underwent fear extinction in a novel context, with a unique cage floor, walls, and scent cues. On day 1 of extinction, there were no differences in freezing time between the treatment groups (**Figure 4M**). 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**).

Fentanyl consumption enhances BLA principal neuron excitability and excitatory balance

Previous studies have found that increased BLA principal neuron excitability contributes to fear extinction deficits [46,47]. Given our observations that fentanyl consumption disrupted normal fear extinction learning, we next investigated BLA principal neuron physiology. All BLA recordings occurred at least 10 days following fear conditioning and 3-5 days into fentanyl abstinence. All mice used for recordings underwent fear conditioning and extinction. We first assessed excitatory/inhibitory (E/I) balance in voltage clamp. We did not observe any significant sex differences in this data

set, so male and female data were combined. There was a significant decrease in sIPSC frequency in the fentanyl group (**Figure 5A & B**) but no change in sISPC amplitude between groups (**Figure 5D**). We observed an increase in sEPSC frequency in the fentanyl group (**Figure 5A & C**), with no change in sEPSC amplitude (**Figure 5E**). Due to the opposing changes in sIPSC and sEPSC frequency observed in the fentanyl group, we found an increased E/I ratio in the fentanyl group (**Figure 5G**). To further assess the relative strength of excitatory and inhibitory inputs, we calculated the synaptic drive ratio for each cell. The synaptic drive ratio is calculated as the frequency of sEPSCs multiplied by the amplitude of sEPSC divided by the frequency of sIPSCs multiplied by the amplitude of sIPSCs. We observed a significant increase in the synaptic drive ratio of the fentanyl group (**Figure 5H**). We next assessed the excitability of BLA principal neurons in current clamp. While holding neurons at -75 mV, we found that the BLA principal neurons from the fentanyl group had a significantly reduced rheobase current (**Figure 5I**). We also tested neuronal responses to hyperpolarizing and depolarizing current injection (**Figure 5F**). BLA neurons from the fentanyl group had a significantly increased number of evoked action potentials per depolarizing current step (**Figure 5J**).

Discussion

Oral fentanyl consumption model

Most studies of opioid consumption have focused on intravenous administration, although other routes of administration, such as vaping or oral ingestion, are increasingly more common for fentanyl [6,7]. 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 fentanyl 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 [9,48]. Alternatively, fentanyl may have an aversive taste at higher concentrations, which may reduce consumption. Notably, we never observed sedative effects of fentanyl in our sleep data, which suggests that consumption is not limited by sedation at these doses. We observed significant sex differences in fentanyl consumption, with females consuming more fentanyl than males, particularly at the 30 µg/mL concentration, however, we have observed that female mice consume more liquid than male mice in general [49,50]. While studies investigating sex differences in oral fentanyl consumption are extremely limited, our work agrees with existing opioid self-administration literature that consistently shows increased opioid self-administration in females versus males [8,13,51-53].

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 [54]. 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 of prescription opioids rather than fentanyl [55,56].

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 [57-59]. 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 [22]. Fentanyl exposure may promote drug-seeking behaviors that get stronger with long-term exposure, which 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.

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. When acutely administered in healthy adults, opioids have been shown to decrease sleep efficiency and increase awakenings [60]. 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 [61,62]. 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 [23,26,63]. Future studies could investigate changes in REM or non-REM sleep due to fentanyl consumption.

Fentanyl consumption alters 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 [64,65]. Our lab has previously demonstrated protracted withdrawal symptoms in mice 6 weeks after naloxone-precipitated morphine withdrawal [19]. We found sex-dependent differences in avoidance behavior after fentanyl administration. Similar to what we observed in protracted morphine withdrawal, female mice that consumed fentanyl spent less time in the surround area of the open field which suggests increased avoidance behavior [19]. 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. 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, and the association between anxiety and opioid use disorder is especially strong in women [66-68]. 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 [69,70]. 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 [71]. 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 [72,73]. 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 [44,45]. We found that 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 not reflect the effects of active opioid exposure, and all fentanyl would be cleared by this time point [71].

The basolateral amygdala is one brain region that is critical for cued fear extinction and reward learning [46,74,75]. We found that BLA principal neurons had greater excitatory inputs and were more excitable following fentanyl consumption. Importantly, previous work has demonstrated that increased BLA principal neuron excitability contributes to fear extinction deficits [76,77]. While it is unclear which specific inputs to BLA

principal neurons were altered, we observed both a decrease in inhibitory inputs and an increase in excitatory inputs. μ-ORs are robustly expressed in GABAergic lateral paracapsular neurons, and μ-OR activation inhibits these cells [78]. Chronic fentanyl consumption may disrupt these GABAergic projections to BLA principal neurons to impair fear extinction [79]. μ-ORs are also expressed in the PFC, which is a major excitatory input to the BLA. While acute μ-OR activation decreases excitatory inputs from the PFC to BLA, chronic fentanyl exposure may alter PFC plasticity to increase excitatory inputs to the BLA [80,81]. While these findings may be important to understanding how fentanyl exposure affects retention of aversive memories, future studies are needed to further investigate molecular and circuit 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, fear extinction, and BLA physiology 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

A.M.D designed and performed experiments, analyzed data, and wrote the manuscript. G.K 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 of the work.

Funding

This work was funded by R01DA049261 (ZAM) and T32AA007573 (AMD).

Competing Interests

Z.A.M is a co-investigator on SBIR grant 1R43DA057749-01 with Epicypher Inc. A.M.D. and G.K. have no competing interests.

Literature Cited

1 Centers for Disease Control, N. C. f. H. S., Office of Communication. *Drug Overdose Deaths in the U.S. Top 100,000 Annually*, <https://www.cdc.gov/nchs/pressroom/nchs_press_releases/2021/20211117.htm> (2021).

2 Ahmad, F. B., Rossen, L. M. & Sutton, P. Provisional drug overdose death counts. *National center for health statistics* **12** (2021).

3 Jeffery, M. M., Stevens, M., D'Onofrio, G. & Melnick, E. R. Fentanyl-Associated Overdose Deaths Outside the Hospital. *The New England journal of medicine* **389**, 87-88 (2023). https://doi.org:10.1056/NEJMc2304991

4 Strang, J. *et al.* Opioid use disorder. *Nature Reviews Disease Primers* **6**, 3 (2020). https://doi.org:10.1038/s41572-019-0137-5

5 McCabe, S. E., Cranford, J. A., Boyd, C. J. & Teter, C. J. Motives, diversion and routes of administration associated with nonmedical use of prescription opioids. *Addictive Behaviors* **32**, 562-575 (2007). https://doi.org:https://doi.org/10.1016/j.addbeh.2006.05.022

6 Young, A. M., Havens, J. R. & Leukefeld, C. G. Route of administration for illicit prescription opioids: a comparison of rural and urban drug users. *Harm reduction journal* **7**, 24 (2010). https://doi.org:10.1186/1477-7517-7-24

7 Daniulaityte, R., Sweeney, K., Ki, S., Doebbeling, B. N. & Mendoza, N. “They say it’s fentanyl, but they honestly look like Perc 30s”: Initiation and use of counterfeit fentanyl pills. *Harm reduction journal* **19**, 52 (2022). https://doi.org:10.1186/s12954-022-00634-4

8 Monroe, S. C. & Radke, A. K. Aversion-resistant fentanyl self-administration in mice. *Psychopharmacology* **238**, 699-710 (2021). https://doi.org:10.1007/s00213-020-05722-6

9 Enga, R. M., Jackson, A., Damaj, M. I. & Beardsley, P. M. Oxycodone physical dependence and its oral self-administration in C57BL/6J mice. *European journal of pharmacology* **789**, 75-80 (2016). https://doi.org:10.1016/j.ejphar.2016.07.006

10 Phillips, A. G. *et al.* Oral prescription opioid-seeking behavior in male and female mice. *Addiction biology* **25**, e12828 (2020). https://doi.org:10.1111/adb.12828

11 Wade, C. L., Schuster, D. J., Domingo, K. M., Kitto, K. F. & Fairbanks, C. A. Supraspinally-administered agmatine attenuates the development of oral fentanyl self-administration. *European journal of pharmacology* **587**, 135-140 (2008). https://doi.org:10.1016/j.ejphar.2008.04.007

12 Klein, L. C. Effects of adolescent nicotine exposure on opioid consumption and neuroendocrine responses in adult male and female rats. *Experimental and clinical psychopharmacology* **9**, 251-261 (2001). https://doi.org:10.1037//1064-1297.9.3.251

13 Klein, L. C., Popke, E. J. & Grunberg, N. E. Sex differences in effects of predictable and unpredictable footshock on fentanyl self-administration in rats. *Experimental and clinical psychopharmacology* **5**, 99-106 (1997). https://doi.org:10.1037//1064-1297.5.2.99

14 Shaham, Y., Klein, L. C., Alvares, K. & Grunberg, N. E. Effect of stress on oral fentanyl consumption in rats in an operant self-administration paradigm. *Pharmacology, biochemistry, and behavior* **46**, 315-322 (1993). https://doi.org:10.1016/0091-3057(93)90359-2
15 Thornton, S. R., Lohmann, A. B., Nicholson, R. A. & Smith, F. L. Fentanyl self-administration in juvenile rats that were tolerant and dependent to fentanyl as infants. *Pharmacology, biochemistry, and behavior* **65**, 563-570 (2000). https://doi.org:10.1016/s0091-3057(99)00262-2
16 Grim, T. W., Park, S. J., Schmid, C. L., Laprairie, R. B., Cameron, M. & Bohn, L. M. The effect of quinine in two bottle choice procedures in C57BL6 mice: Opioid preference, somatic withdrawal, and pharmacokinetic outcomes. *Drug and alcohol dependence* **191**, 195-202 (2018). https://doi.org:10.1016/j.drugalcdep.2018.05.034
17 Shaham, Y., Alvares, K., Nespor, S. M. & Grunberg, N. E. Effect of stress on oral morphine and fentanyl self-administration in rats. *Pharmacology, biochemistry, and behavior* **41**, 615-619 (1992). https://doi.org:10.1016/0091-3057(92)90382-p
18 Bedard, M. L. *et al.* Probing different paradigms of morphine withdrawal on sleep behavior in male and female C57BL/6J mice. *Behavioural brain research* **448**, 114441 (2023). https://doi.org:10.1016/j.bbr.2023.114441
19 Bravo, I. M. *et al.* Divergent behavioral responses in protracted opioid withdrawal in male and female C57BL/6J mice. *The European journal of neuroscience* **51**, 742-754 (2020). https://doi.org:10.1111/ejn.14580
20 Luster, B. R. *et al.* Inhibitory transmission in the bed nucleus of the stria terminalis in male and female mice following morphine withdrawal. *Addiction biology* **25**, e12748 (2020). https://doi.org:10.1111/adb.12748
21 Bedard, M. L. *et al.* Probing different paradigms of morphine withdrawal on sleep behavior in male and female C57BL/6J mice. *bioRxiv* (2023). https://doi.org:10.1101/2022.04.06.487380
22 Coffey, A. A., Guan, Z., Grigson, P. S. & Fang, J. Reversal of the sleep-wake cycle by heroin self-administration in rats. *Brain research bulletin* **123**, 33-46 (2016). https://doi.org:10.1016/j.brainresbull.2015.09.008
23 Dimsdale, J. E., Norman, D., DeJardin 1, D. & Wallace, M. S. The effect of opioids on sleep architecture. *Journal of clinical sleep medicine* **3**, 33-36 (2007).
24 Hartwell, E. E., Pfeifer, J. G., McCauley, J. L., Moran-Santa Maria, M. & Back, S. E. Sleep disturbances and pain among individuals with prescription opioid dependence. *Addict Behav* **39**, 1537-1542 (2014). https://doi.org:10.1016/j.addbeh.2014.05.025
25 Huhn, A. S. & Finan, P. H. Sleep disturbance as a therapeutic target to improve opioid use disorder treatment. *Experimental and clinical psychopharmacology* **30**, 1024-1035 (2022). https://doi.org:10.1037/pha0000477
26 Walder, B., Tramèr, M. R. & Blois, R. The effects of two single doses of tramadol on sleep: a randomized, cross-over trial in healthy volunteers. *European Journal of Anaesthesiology* **18**, 36-42 (2001). https://doi.org:https://doi.org/10.1046/j.1365-2346.2001.00772.x
27 Zebadua Unzaga, D., Bustamante, C., Thibert, M. & Baghdoyan, H. Fentanyl and Morphine Cause Dose-Dependent Sleep Disruption in C57BL/6J (B6) Mice. *The*

FASEB Journal **35** (2021). https://doi.org:https://doi.org/10.1096/fasebj.2021.35.S1.01706

28 Donohue, K. D., Medonza, D. C., Crane, E. R. & O'Hara, B. F. Assessment of a non-invasive high-throughput classifier for behaviours associated with sleep and wake in mice. *Biomedical engineering online* **7**, 14 (2008). https://doi.org:10.1186/1475-925x-7-14

29 Flores, A. E. *et al.* Pattern recognition of sleep in rodents using piezoelectric signals generated by gross body movements. *IEEE transactions on bio-medical engineering* **54**, 225-233 (2007). https://doi.org:10.1109/tbme.2006.886938

30 Mang, G. M., Nicod, J., Emmenegger, Y., Donohue, K. D., O'Hara, B. F. & Franken, P. Evaluation of a piezoelectric system as an alternative to electroencephalogram/ electromyogram recordings in mouse sleep studies. *Sleep* **37**, 1383-1392 (2014). https://doi.org:10.5665/sleep.3936

31 Bravo, I., Bluitt, M. & McElligott, Z. Examining opioid withdrawal scoring and adaptation of global scoring systems to male and female C57BL/6J mice. *bioRxiv*, 2021.2010.2011.463944 (2021). https://doi.org:10.1101/2021.10.11.463944

32 Torruella-Suárez, M. L. *et al.* Manipulations of Central Amygdala Neurotensin Neurons Alter the Consumption of Ethanol and Sweet Fluids in Mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **40**, 632-647 (2020). https://doi.org:10.1523/jneurosci.1466-19.2019

33 Dao, N. C. *et al.* Forced Abstinence From Alcohol Induces Sex-Specific Depression-Like Behavioral and Neural Adaptations in Somatostatin Neurons in Cortical and Amygdalar Regions. *Frontiers in behavioral neuroscience* **14** (2020). https://doi.org:10.3389/fnbeh.2020.00086

34 Downs, A. M., Catavero, C. M., Kasten, M. R. & McElligott, Z. A. Tauopathy and alcohol consumption interact to alter locus coeruleus excitatory transmission and excitability in male and female mice. *Alcohol (Fayetteville, N.Y.)* **107**, 97-107 (2023). https://doi.org:10.1016/j.alcohol.2022.08.008

35 Downs, A. M. & McElligott, Z. A. Noradrenergic circuits and signaling in substance use disorders. *Neuropharmacology* **208**, 108997 (2022). https://doi.org:10.1016/j.neuropharm.2022.108997

36 Faccidomo, S. *et al.* Calcium-permeable AMPA receptor activity and GluA1 trafficking in the basolateral amygdala regulate operant alcohol self-administration. *Addiction biology* **26**, e13049 (2021). https://doi.org:10.1111/adb.13049

37 Bedard, M. L. *et al.* Probing different paradigms of morphine withdrawal on sleep behavior in male and female C57BL/6J mice. **448**, 114441 (2023).

38 Carlson, K. R. Taste vs. CNS effects in voluntary oral opiate intake: studies with a novel device and technique. *Pharmacology, biochemistry, and behavior* **34**, 419-423 (1989). https://doi.org:10.1016/0091-3057(89)90336-5

39 Alipio, J. B. *et al.* Enduring consequences of perinatal fentanyl exposure in mice. *Addiction biology* **26**, e12895 (2021). https://doi.org:10.1111/adb.12895

40 Choi, J. *et al.* The Role of Reproductive Hormones in Sex Differences in Sleep Homeostasis and Arousal Response in Mice. *Front Neurosci* **15**, 739236 (2021). https://doi.org:10.3389/fnins.2021.739236

41 Destoop, M., Morrens, M., Coppens, V. & Dom, G. Addiction, Anhedonia, and Comorbid Mood Disorder. A Narrative Review. *Frontiers in Psychiatry* **10** (2019). https://doi.org:10.3389/fpsyt.2019.00311
42 Garfield, J. B. B. *et al.* Evidence that anhedonia is a symptom of opioid dependence associated with recent use. *Drug and alcohol dependence* **177**, 29-38 (2017). https://doi.org:https://doi.org/10.1016/j.drugalcdep.2017.03.012
43 Koob, G. F. & Volkow, N. D. Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* **3**, 760-773 (2016). https://doi.org:10.1016/s2215-0366(16)00104-8
44 Gu, C. *et al.* Chronic Morphine Selectively Impairs Cued Fear Extinction in Rats: Implications for Anxiety Disorders Associated with Opiate Use. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **33**, 666-673 (2008). https://doi.org:10.1038/sj.npp.1301441
45 Pennington, Z. T. *et al.* Chronic opioid pretreatment potentiates the sensitization of fear learning by trauma. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **45**, 482-490 (2020). https://doi.org:10.1038/s41386-019-0559-5
46 Gale, G. D. *et al.* Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 3810-3815 (2004). https://doi.org:10.1523/jneurosci.4100-03.2004
47 Sharp, B. M. Basolateral amygdala and stress-induced hyperexcitability affect motivated behaviors and addiction. *Translational psychiatry* **7**, e1194-e1194 (2017). https://doi.org:10.1038/tp.2017.161
48 Slivicki, R. A. *et al.* Oral oxycodone self-administration leads to features of opioid misuse in male and female mice. *Addiction biology* **28**, e13253 (2023). https://doi.org:10.1111/adb.13253
49 Catavero, C. M., Marsh, A. E., Downs, A. M., Teklezghi, A. T., Cohen, T. J. & McElligott, Z. A. Effects of Long-Term Alcohol Consumption on Behavior in the P301S (Line PS19) Tauopathy Mouse Model. *bioRxiv*, 2022.2007.2012.499737 (2022). https://doi.org:10.1101/2022.07.12.499737
50 Gereau, G. B. *et al.* GABA Release From Central Amygdala Neurotensin Neurons Differentially Modulates Reward and Consummatory Behavior in Male and Female Mice. *bioRxiv* (2023). https://doi.org:10.1101/2023.09.14.557768
51 Carroll, M. E., Campbell, U. C. & Heideman, P. Ketoconazole suppresses food restriction-induced increases in heroin self-administration in rats: sex differences. *Experimental and clinical psychopharmacology* **9**, 307-316 (2001). https://doi.org:10.1037//1064-1297.9.3.307
52 Cicero, T. J., Aylward, S. C. & Meyer, E. R. Gender differences in the intravenous self-administration of mu opiate agonists. *Pharmacology, biochemistry, and behavior* **74**, 541-549 (2003). https://doi.org:10.1016/s0091-3057(02)01039-0
53 Mavrikaki, M., Pravetoni, M., Page, S., Potter, D. & Chartoff, E. Oxycodone self-administration in male and female rats. *Psychopharmacology* **234**, 977-987 (2017).

54 Bedard, M. L. *et al.* Xylazine is an agonist at kappa opioid receptors and exhibits sex-specific responses to naloxone administration. *bioRxiv*, 2023.2009.2008.556914 (2023). https://doi.org:10.1101/2023.09.08.556914
55 Brady, K. T. & Randall, C. L. Gender differences in substance use disorders. *Psychiatr Clin North Am* **22**, 241-252 (1999). https://doi.org:10.1016/s0193-953x(05)70074-5
56 Kosten, T. R., Rounsaville, B. J. & Kleber, H. D. Ethnic and gender differences among opiate addicts. *Int J Addict* **20**, 1143-1162 (1985). https://doi.org:10.3109/10826088509056356
57 Koek, W., France, C. P. & Javors, M. A. Morphine-induced motor stimulation, motor incoordination, and hypothermia in adolescent and adult mice. *Psychopharmacology* **219**, 1027-1037 (2012). https://doi.org:10.1007/s00213-011-2432-z
58 Kennedy, B. C., Panksepp, J. B., Wong, J. C., Krause, E. J. & Lahvis, G. P. Age-dependent and strain-dependent influences of morphine on mouse social investigation behavior. *Behav Pharmacol* **22**, 147-159 (2011). https://doi.org:10.1097/FBP.0b013e328343d7dd
59 Maldonado, R., Saiardi, A., Valverde, O., Samad, T. A., Roques, B. P. & Borrelli, E. Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**, 586-589 (1997). https://doi.org:10.1038/41567
60 Xiao, L. *et al.* Nocturnal sleep architecture disturbances in early methadone treatment patients. **179**, 91-95 (2010).
61 Rosen, I. M. *et al.* Chronic Opioid Therapy and Sleep: An American Academy of Sleep Medicine Position Statement. *Journal of Clinical Sleep Medicine* **15**, 1671-1673 (2019). https://doi.org:doi:10.5664/jcsm.8062
62 Baldassarri, S. R. *et al.* Correlates of sleep quality and excessive daytime sleepiness in people with opioid use disorder receiving methadone treatment. *Sleep Breath* **24**, 1729-1737 (2020). https://doi.org:10.1007/s11325-020-02123-z
63 Pickworth, W. B., Neidert, G. L. & Kay, D. C. Morphinelike arousal by methadone during sleep. *Clinical Pharmacology & Therapeutics* **30**, 796-804 (1981). https://doi.org:https://doi.org/10.1038/clpt.1981.240
64 Hyman, S. M., Fox, H., Hong, K. I., Doebrick, C. & Sinha, R. Stress and drug-cue-induced craving in opioid-dependent individuals in naltrexone treatment. *Experimental and clinical psychopharmacology* **15**, 134-143 (2007). https://doi.org:10.1037/1064-1297.15.2.134
65 Kreek, M. J. & Koob, G. F. Drug dependence: stress and dysregulation of brain reward pathways. *Drug and alcohol dependence* **51**, 23-47 (1998). https://doi.org:10.1016/s0376-8716(98)00064-7
66 McHugh, R. K., Votaw, V. R., Sugarman, D. E. & Greenfield, S. F. Sex and gender differences in substance use disorders. *Clin Psychol Rev* **66**, 12-23 (2018). https://doi.org:10.1016/j.cpr.2017.10.012
67 Langdon, K. J., Dove, K. & Ramsey, S. Comorbidity of opioid-related and anxiety-related symptoms and disorders. *Curr Opin Psychol* **30**, 17-23 (2019). https://doi.org:10.1016/j.copsyc.2018.12.020

68 Leung, J. *et al.* Mood and Anxiety Symptoms in Persons Taking Prescription Opioids: A Systematic Review with Meta-Analyses of Longitudinal Studies. *Pain Medicine* **23**, 1442-1456 (2022). https://doi.org:10.1093/pm/pnac029
69 Koob, G. F. The Dark Side of Addiction: The Horsley Gantt to Joseph Brady Connection. *J Nerv Ment Dis* **205**, 270-272 (2017). https://doi.org:10.1097/nmd.0000000000000551
70 Kiluk, B. D., Yip, S. W., DeVito, E. E., Carroll, K. M. & Sofuoglu, M. Anhedonia as a key clinical feature in the maintenance and treatment of opioid use disorder. *Clin Psychol Sci* **7**, 1190-1206 (2019). https://doi.org:10.1177/2167702619855659
71 Kalvass, J. C., Olson, E. R., Cassidy, M. P., Selley, D. E. & Pollack, G. M. Pharmacokinetics and pharmacodynamics of seven opioids in P-glycoprotein-competent mice: assessment of unbound brain EC50,u and correlation of in vitro, preclinical, and clinical data. *The Journal of pharmacology and experimental therapeutics* **323**, 346-355 (2007). https://doi.org:10.1124/jpet.107.119560
72 Roberts, N. P., Roberts, P. A., Jones, N. & Bisson, J. I. Psychological interventions for post-traumatic stress disorder and comorbid substance use disorder: A systematic review and meta-analysis. *Clinical Psychology Review* **38**, 25-38 (2015). https://doi.org:https://doi.org/10.1016/j.cpr.2015.02.007
73 Fareed, A., Eilender, P., Haber, M., Bremner, J., Whitfield, N. & Drexler, K. Comorbid Posttraumatic Stress Disorder and Opiate Addiction: A Literature Review. *Journal of addictive diseases* **32**, 168-179 (2013). https://doi.org:10.1080/10550887.2013.795467
74 Quirk, G. J., Russo, G. K., Barron, J. L. & Lebron, K. The Role of Ventromedial Prefrontal Cortex in the Recovery of Extinguished Fear. *The Journal of Neuroscience* **20**, 6225-6231 (2000). https://doi.org:10.1523/jneurosci.20-16-06225.2000
75 Wassum, K. M. & Izquierdo, A. The basolateral amygdala in reward learning and addiction. *Neurosci Biobehav Rev* **57**, 271-283 (2015). https://doi.org:10.1016/j.neubiorev.2015.08.017
76 Johansen, J. P. *et al.* Optical activation of lateral amygdala pyramidal cells instructs associative fear learning. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12692-12697 (2010). https://doi.org:10.1073/pnas.1002418107
77 Gore, F. *et al.* Neural Representations of Unconditioned Stimuli in Basolateral Amygdala Mediate Innate and Learned Responses. *Cell* **162**, 134-145 (2015). https://doi.org:10.1016/j.cell.2015.06.027
78 Ronström, J. W. *et al.* Opioid-Induced Reductions in Amygdala Lateral Paracapsular GABA Neuron Circuit Activity. *International journal of molecular sciences* **24** (2023). https://doi.org:10.3390/ijms24031929
79 Skelly, M. J., Chappell, A. M., Ariwodola, O. J. & Weiner, J. L. Behavioral and neurophysiological evidence that lateral paracapsular GABAergic synapses in the basolateral amygdala contribute to the acquisition and extinction of fear learning. *Neurobiology of learning and memory* **127**, 10-16 (2016). https://doi.org:10.1016/j.nlm.2015.11.006

80 Baldo, B. A. Prefrontal Cortical Opioids and Dysregulated Motivation: A Network Hypothesis. *Trends in neurosciences* **39**, 366-377 (2016). https://doi.org:10.1016/j.tins.2016.03.004

81 Rosen, L. G., Zunder, J., Renard, J., Fu, J., Rushlow, W. & Laviolette, S. R. Opiate Exposure State Controls a D2-CaMKIIα-Dependent Memory Switch in the Amygdala-Prefrontal Cortical Circuit. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **41**, 847-857 (2016). https://doi.org:10.1038/npp.2015.211

Figures

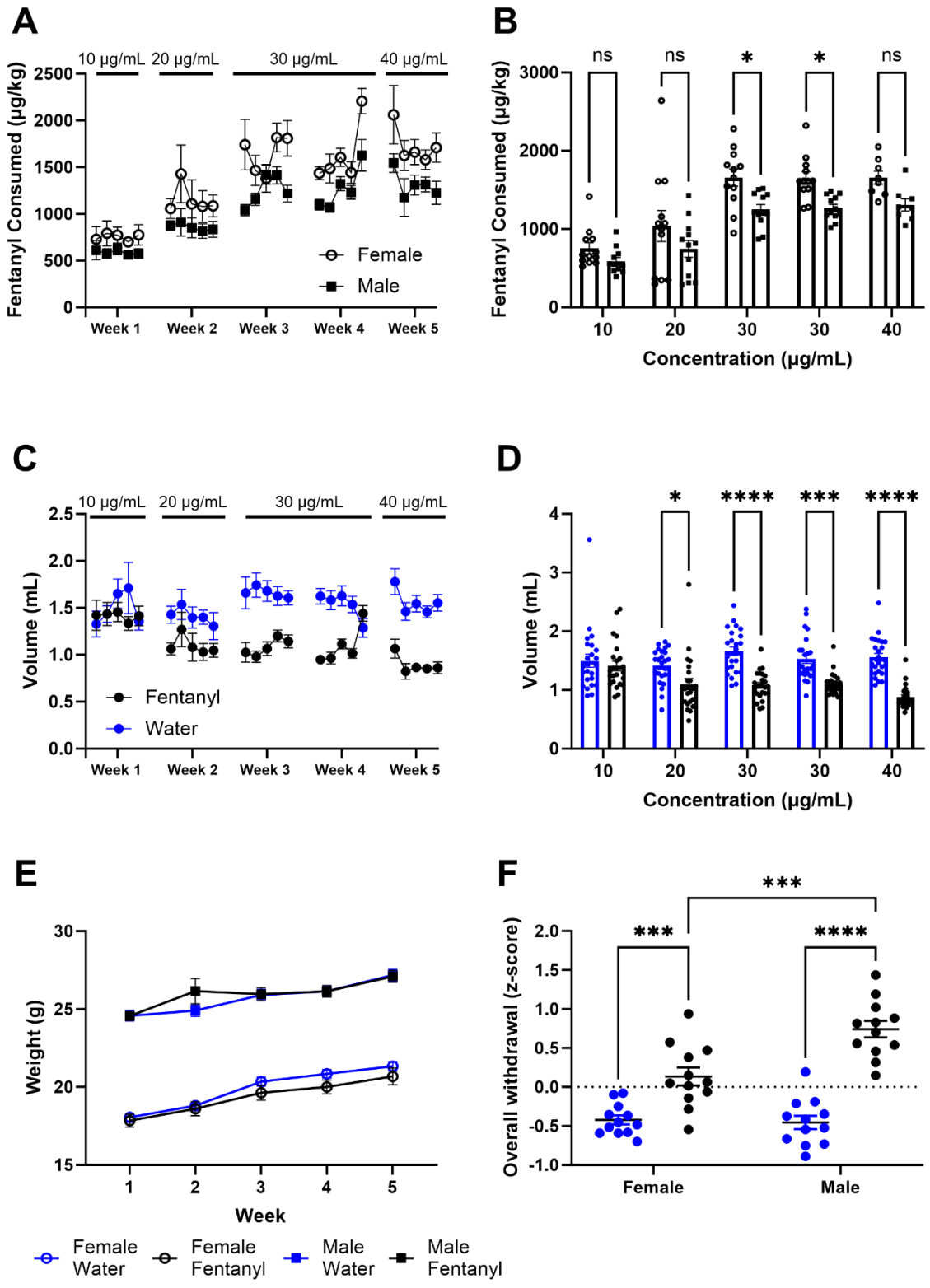
A
10 µg/mL
20 µg/mL
30 µg/mL
40 µg/mL
Fentanyl Consumed (µg/kg)
Week 1
Week 2
Week 3
Week 4
Week 5
Female
Male
B
ns
ns
*
*
ns
Fentanyl Consumed (µg/kg)
Concentration (µg/mL)
C
Volume (mL)
Fentanyl
Water
D
*
****
***
****
Volume (mL)
Concentration (µg/mL)
E
Weight (g)
Week
Female Water
Female Fentanyl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

Figure 1. Volume and fentanyl consumed throughout 5-week fentanyl drinking paradigm and overall withdrawal behavior in female and male mice. **A.** Fentanyl consumed (µg/mL) by day in male and female mice (main effect of dose, $F_{4,102} = 29.08$, $p < 0.0001$). **B.** Average fentanyl consumed per week in male and female mice. Females consumed more fentanyl than males, especially at the 30 µg/mL dose (main effect of sex, $F_{1,102} = 24.1$, $p < 0.0001$, Šídák's multiple comparison test, 30 µg/mL, $p = 0.019$, $p = 0.034$). **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 (time x treatment interaction, $F_{4,184} = 6.86$, $p < 0.0001$; main effect of treatment, $F_{1,46} = 39.52$, $p < 0.0001$). **E.** Weight across 5-week paradigm with no differences observed between treatment groups (main effect of time, $F_{2.08,9.29} = 70.5$, $p < 0.0001$; main effect of treatment, $F_{1,44} = 0.18$, $p = 0.67$). **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 (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 (sex x treatment interaction, $F_{1,44} = 11.49$, $p = 0.0015$; main effect of sex, $F_{1,44} = 9.33$, $p = 0.0038$). Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

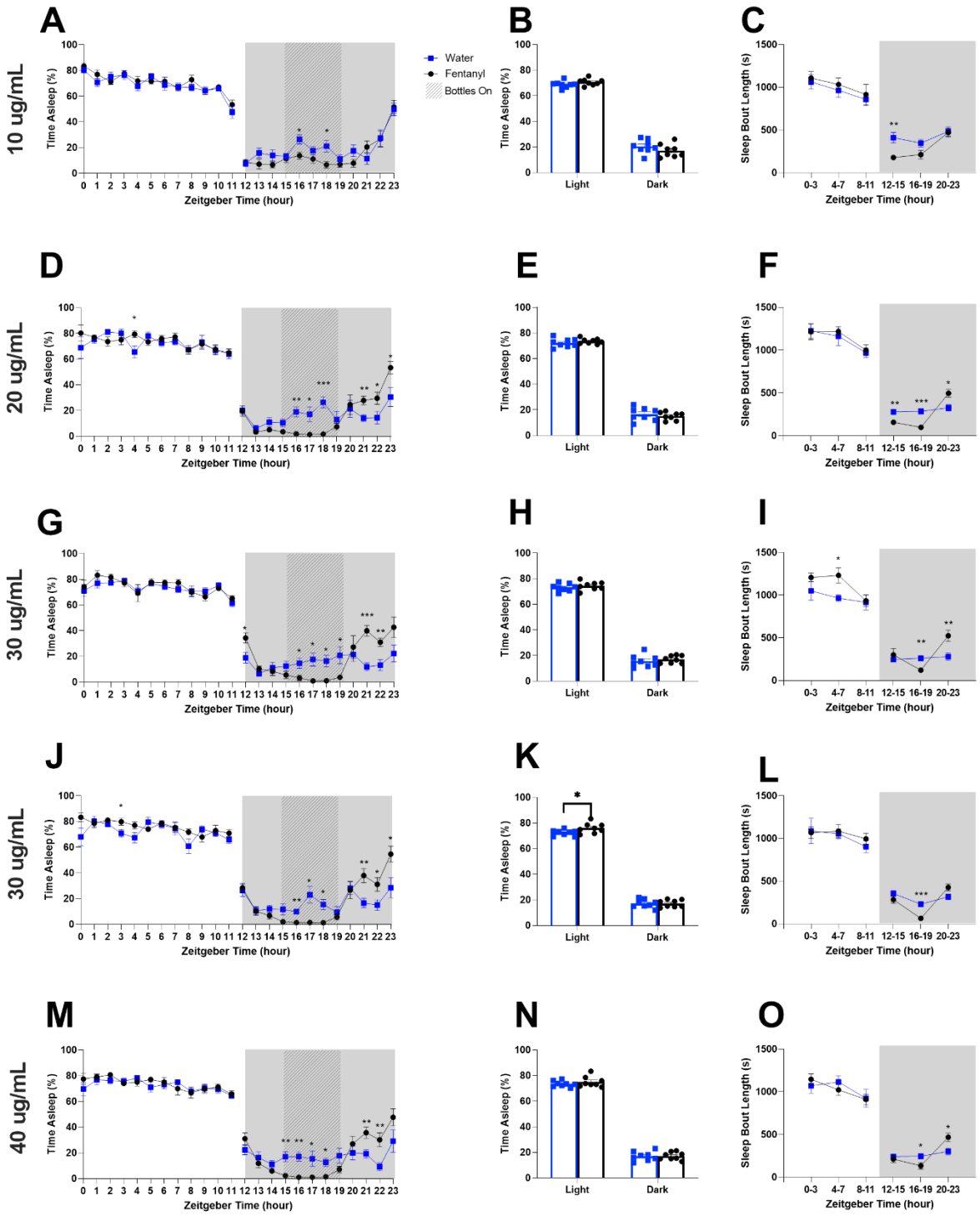

Figure 2. Long-term fentanyl consumption leads to changes in sleep behavior in male mice. **A, D, G, J, M.** Time spent asleep by hour in the light (unshaded) and dark (shaded) periods at each dose. Textured region shows the hours at which the fentanyl bottles were placed on cages, during which the fentanyl group slept less than the water group. At the 20 µg/mL dose and greater (**D, G, J, M**), the fentanyl group slept less 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 had 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): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

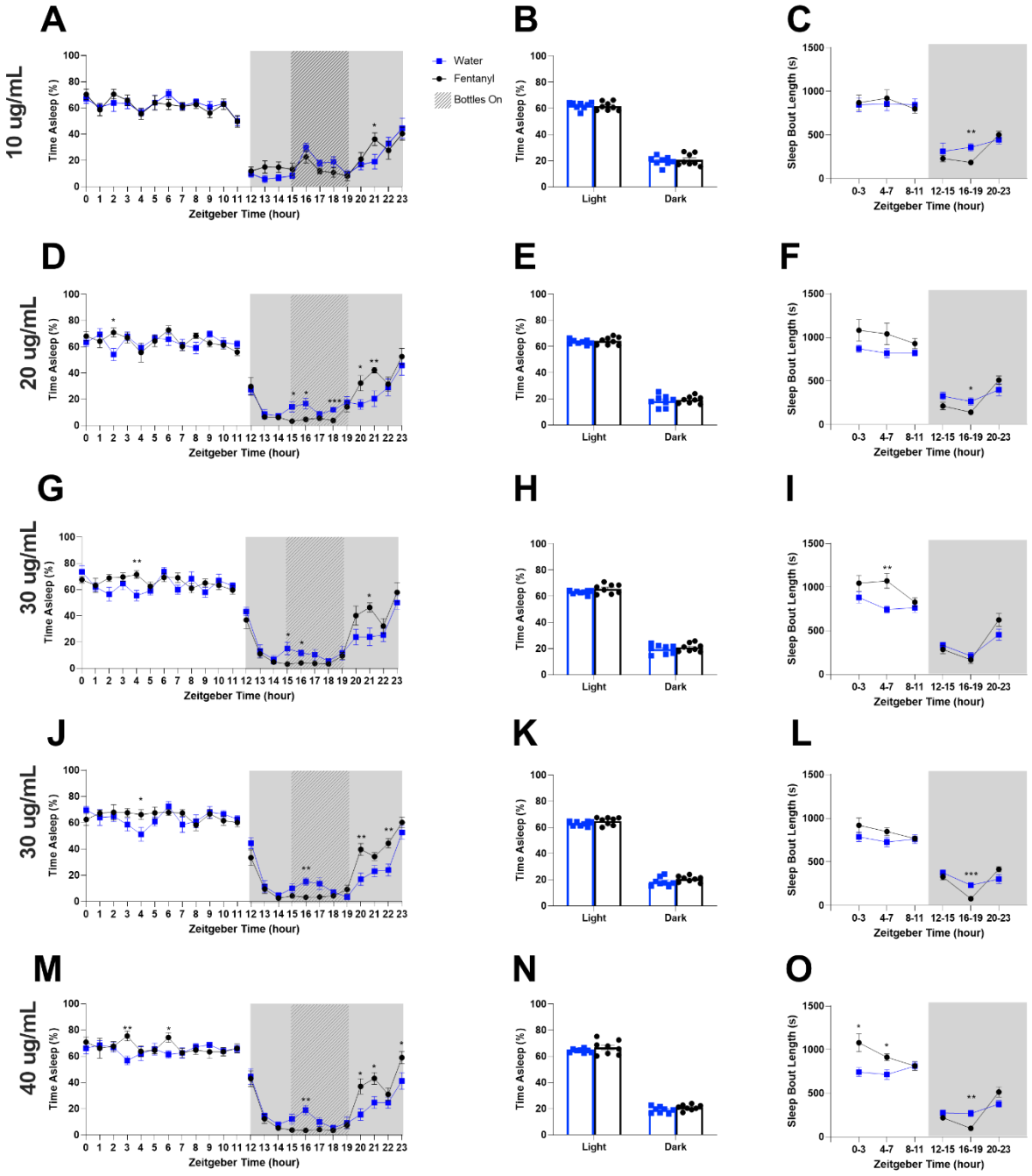

Figure 3. Long-term fentanyl consumption leads to changes in sleep behavior in female mice. **A, D, G, J, M.** Time spent asleep by hour in the light (unshaded) and dark (shaded) periods at each dose. Textured region shows the hours at which the fentanyl bottles were placed on cages. The fentanyl group 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): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

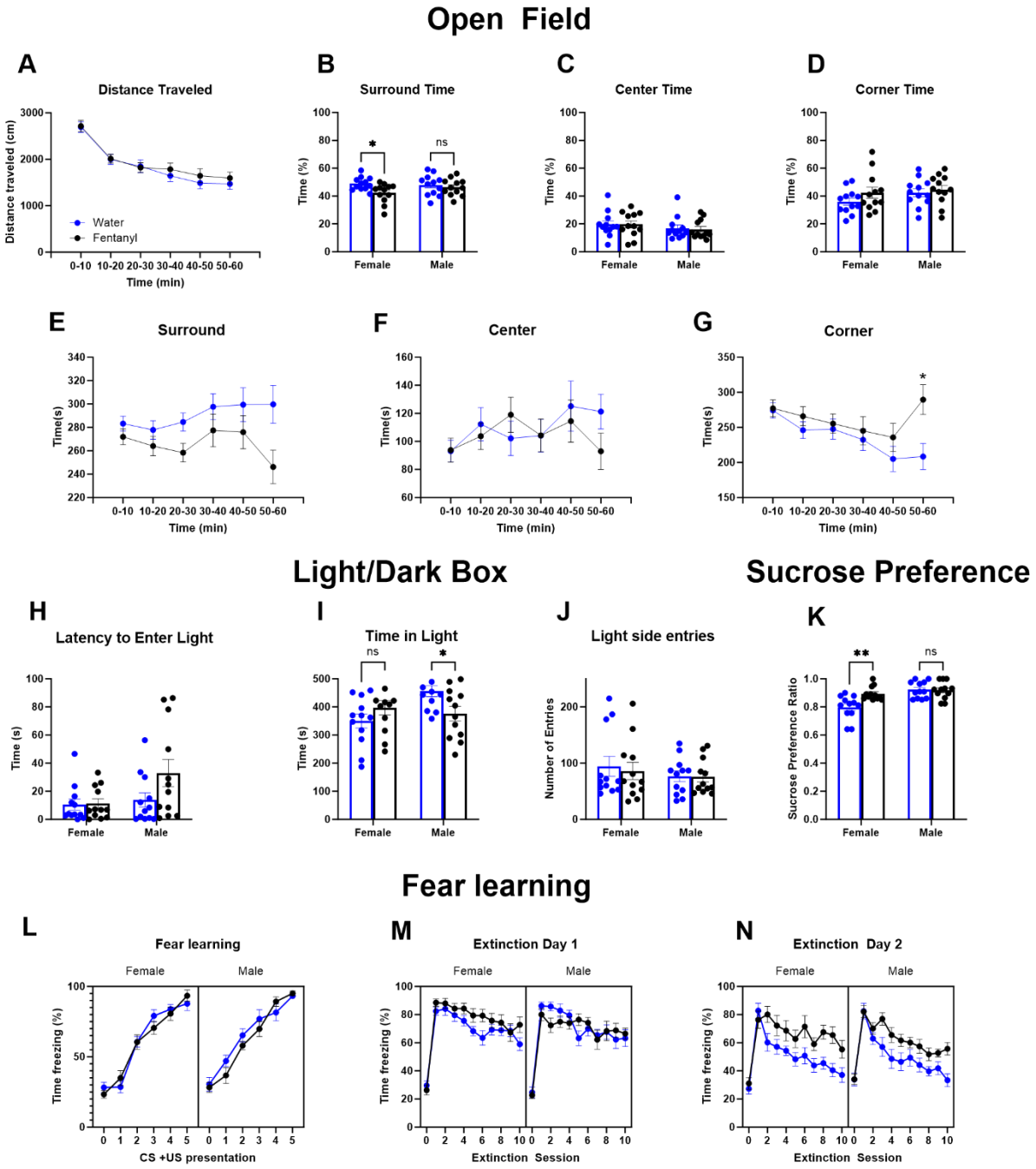

Figure 4. Behavioral assays following fentanyl drinking paradigm in male and female mice. **A, B, C, D, E, F, G.** Show behavior in the open field assay for locomotor activity. **A.** Treatment groups showed no differences in total distance traveled when binned in 10-minute increments across the 60-minute assay (main effect of treatment, $F_{1,46}$ = 0.27, p = 0.61). **B.** Females showed decreased percent time spent in the surround in the fentanyl group, while males showed no differences across treatment groups (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). **C, D.** No differences were observed in the percent time spent in the

center or corners between treatment groups for either sex (main effect of treatment, center: p=0.8648, corners: p=0.1793). **E.** Fentanyl groups spent relatively less time in the surround region towards the end of the assay (main effect of treatment, $F_{1,46} = 5.01$, $p = 0.03$). **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 (treatment x time interaction, $F_{5,230} = 4.15$, $p = 0.001$, Šídák's multiple comparisons test, $p = 0.036$). **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 (main effect of treatment, $F_{1,44} = 2.77$, p=0.1035). **I.** The male fentanyl group spent less time in the light overall than the male water group, while females did not show significant differences (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). **J.** For both sexes, there were no differences in the number of times subjects entered the light side (main effect of treatment, $F_{1,44} = 0.12$, p=0.7307). **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 (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). **L, M, N.** Show the fear conditioning assay. **L.** Percent time spent freezing during fear learning was consistent across treatment groups for both sexes (main effect of treatment, $F_{1,44} = 0.50$, $p = 0.49$). 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 (main effect of treatment, $F_{1,44} = 1.37$, $p = 0.25$). **N.** On the second day, the fentanyl groups for both sexes showed significantly more time freezing across all cues (treatment x tone presentation interaction effect, $F_{10.440} = 3.01$, $p = 0.001$, main effect of treatment, $F_{1,44} = 15.6$, $p = 0.0003$). Each point represents the mean ± standard error of the mean (SEM): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

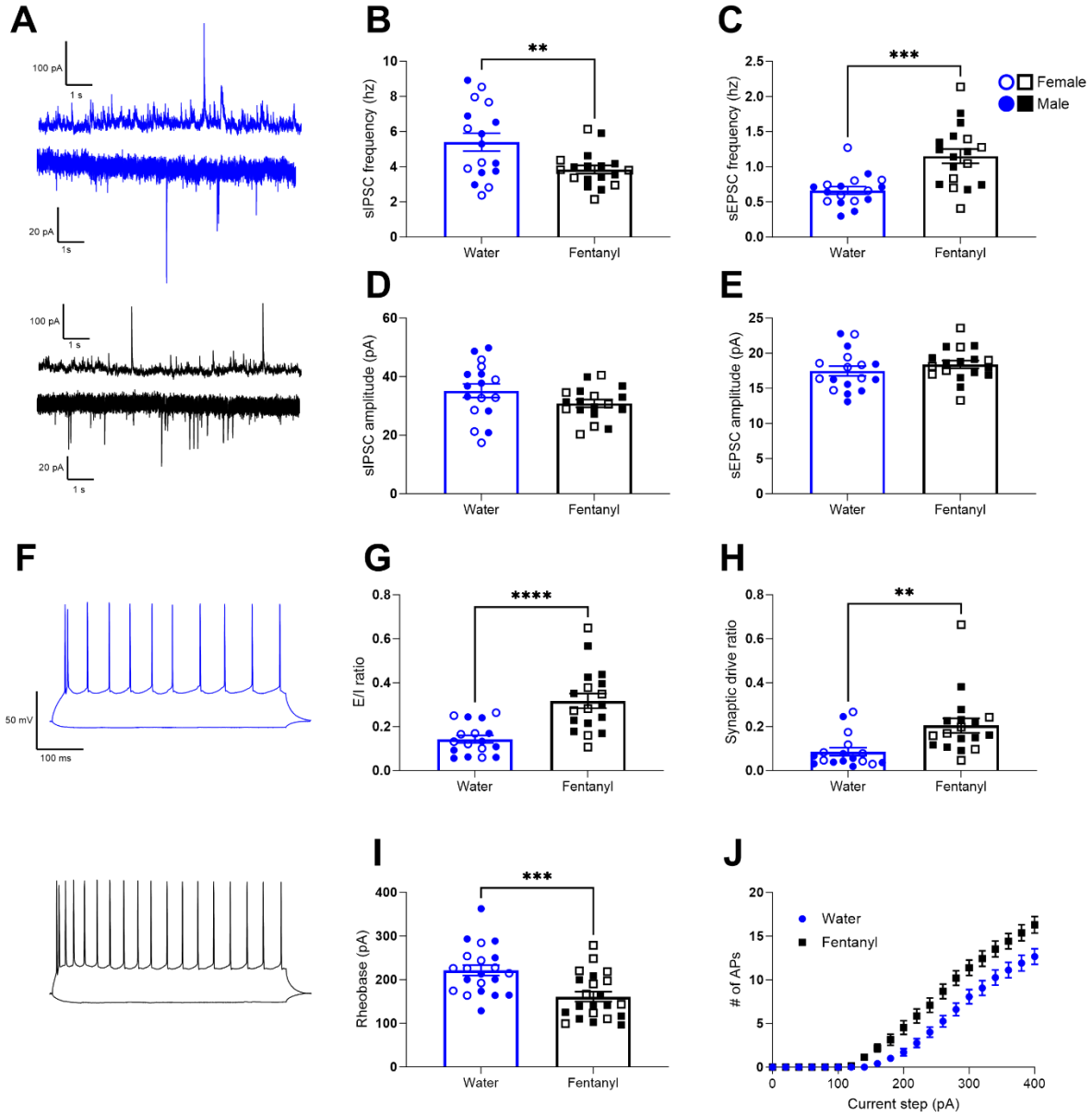

Figure 5. BLA neuron principal neuron physiology is altered following fentanyl self-administration. **A.** Representative sIPSCs traces (top) and sEPSCs traces (bottom) from water drinking (blue) and fentanyl drinking (black) mice. **B**. sIPSC frequency is reduced following fentanyl administration ($t(33) = 2.814$, $p = 0.008$). **C**. sEPSC frequency is increased following fentanyl consumption ($t(33) = 4.126$, $p = 0.0002$). sIPSC amplitude (**D**) ($t(33) = 1.636$, $p = 0.111$) and sEPSC amplitude (**E**) ($t(33) = 1.052$, $p = 0.301$) are unchanged following fentanyl consumption. **F.** Representative traces of evoked action potentials following -200 pA and 400 pA current injection. **G**. Excitation/Inhibition (E/I) ratio is increased following fentanyl consumption ($t(33) = 4.513$, $p < 0.0001$). **H**. Synaptic drive is increased following fentanyl consumption ($t(33) = 3.125$, $p = 0.004$). **I**.

Rheobase current is decreased following fentanyl consumption ($t(41) = 3.677$,, $p = 0.0007$). **J.** Evoked action potential firing following positive current injection is increased following fentanyl consumption (current step x treatment interaction effect, $F_{20,780} = 6.45$, $p < 0.0001$). Open symbols represent females and closed symbols represent males. 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

Supplemental Methods

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 experimental manipulations.

Opioid withdrawal behaviors

All animals were injected with 1 mg/kg naloxone following 4-5 hours of free access to 30 µg/mL fentanyl in water or water as a control. Immediately after injection, mice were placed in an open arena and withdrawal behaviors were assessed for 10 mins. The withdrawal behaviors evaluated included: escape jumps, paw tremors, jaw tremors, abnormal postures, wet dog shakes, grooming behaviors, and fecal boli number. Data was converted to a z-score for each behavior and an average z-score of all behaviors together was calculated to generate a global withdrawal score for each mouse [31].

Avoidance Assays

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 the 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).

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).

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 with one another 12 hours into the drinking period to account for side preference. The bottles were 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.

Brain slice preparation

Briefly, mice were deeply anesthetized with isoflurane, decapitated, and brains were removed and placed into ice-cold sucrose aCSF [in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 NaH_2PO_4. 10 glucose, 26 $NaHCO_3$] oxygenated with 95% O_2 5% CO_2 for slicing. Brains were sliced coronally at 200-300 μm using a Leica VT1000 vibratome (Germany). Slices were then transferred to a holding container with oxygenated aCSF [in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$. 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$] at 32 °C for at least 45 minutes. Slices were then transferred to a recording chamber and perfused with oxygenated aCSF [in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$. 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$] at 30 °C at a constant rate of 2 mL/min.

Whole-cell recordings

Recording electrodes (3-5 MΩ) were pulled on a P-97 Micropipette Puller (Sutter Instruments). Current-clamp recordings for excitability were conducted with potassium-gluconate intercellular recording solution [in mM: 135 gluconic acid potassium, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP]. Voltage-clamp recordings for excitatory/inhibitory balance were conducted with cesium methane sulfonate intercellular recording solution [in mM: 135 Cs-methane sulfonate, 10 KCl, 10 HEPES, 1 $MgCl_2$. 0.2 EGTA, 4 Na_2ATP, 0.3 Na_2GTP, 20 creatine phosphate]. All signals were acquired using an Axon Multiclamp 700B (Molecular Devices Sunnyvale, CA). Input

resistance, holding current, and access resistance were continuously monitored throughout the experiment. Cells in which access resistance changed greater than 20% were excluded from analysis. 1-3 cells were recorded from each animal for each set of experiments.

BLA principal neurons were identified by their anatomical location, low membrane resistance < 50 MΩ and high capacitance > 100 pF. Spontaneous glutamate-mediated excitatory postsynaptic currents (sEPSCs) were acquired in voltage clamp at a -55 mV holding potential. Spontaneous GABA-mediated inhibitory postsynaptic currents (sIPSCs) were acquired from the same cells at a +10 mV holding potential. Excitability studies were performed in current clamp mode with current injected to hold cells to a common membrane potential of -75 mV to account for inter-cell variability. Rheobase currents were determined with a series of current ramps (0-100 pA at 120 pA/s, 100-220 pA at 120 pA/s, and 200-320 at 120 pA/s). Excitability was then assessed with current injection ranging from -100 to +400 pA at 20 pA intervals, and the number of action potentials at each current step were counted.

Supplemental Results

Opioid withdrawal behaviors

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, $F_{1,44} = 7.62$, $p = 0.008$).

Baseline Sleep Behavior

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.

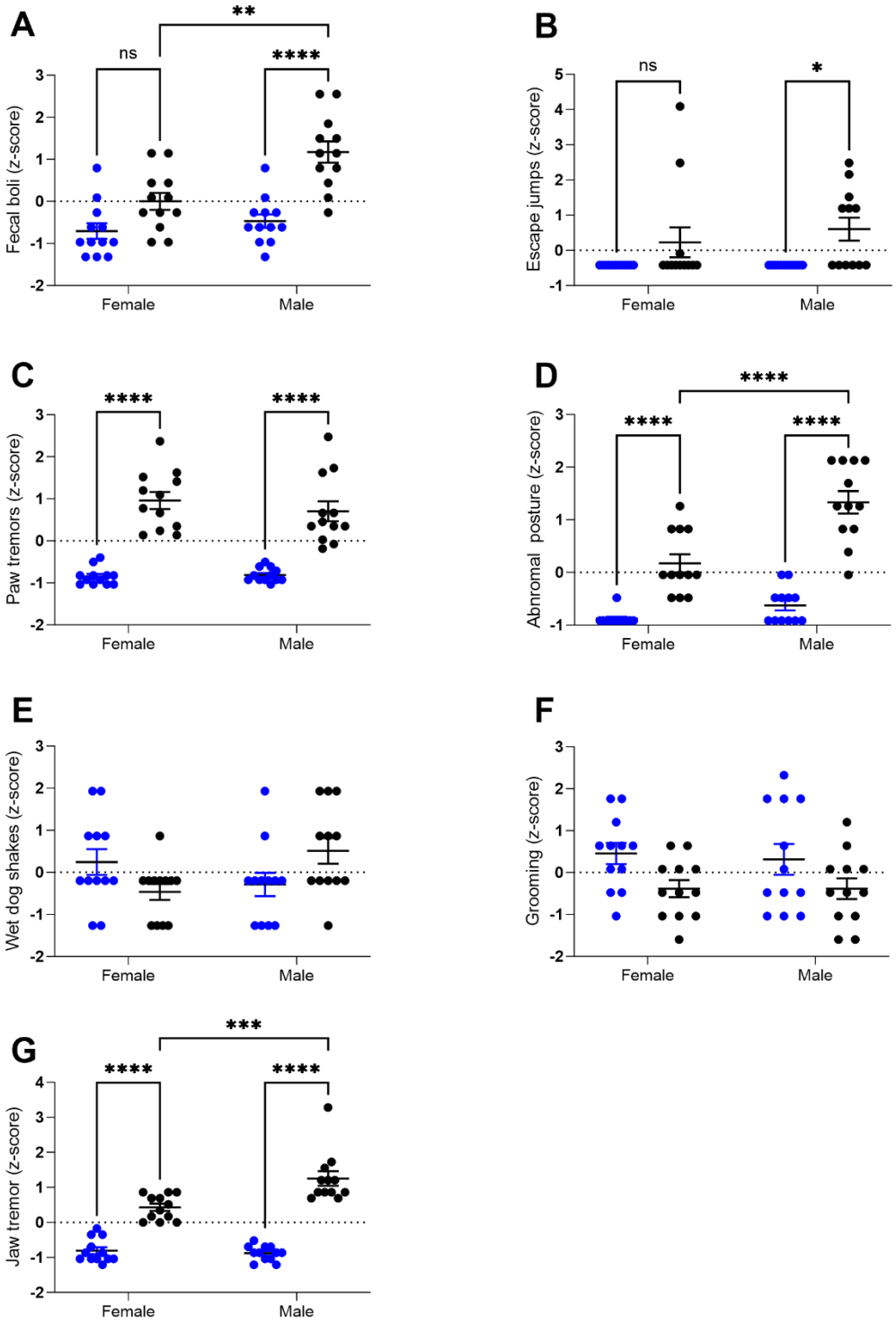

Figure S1. Long-term fentanyl consumption shows sex differences in withdrawal behaviors. **A** Male fentanyl group produced more fecal boli than both male water group and female fentanyl group. **B** Only males exhibited more escape jumps in the fentanyl group. Females showed no differences between treatment 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): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

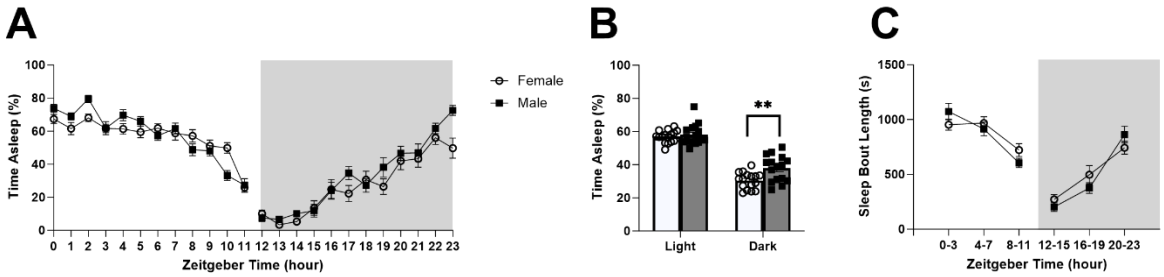

Figure S2. Female and male mice show differences in baseline sleep. **A** Females slept less in the earlier hours and more in the latter hours of the light periods. Females also slept less in the dark periods, especially in the latter hours. **B** Females slept less, on average, during the dark periods 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): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

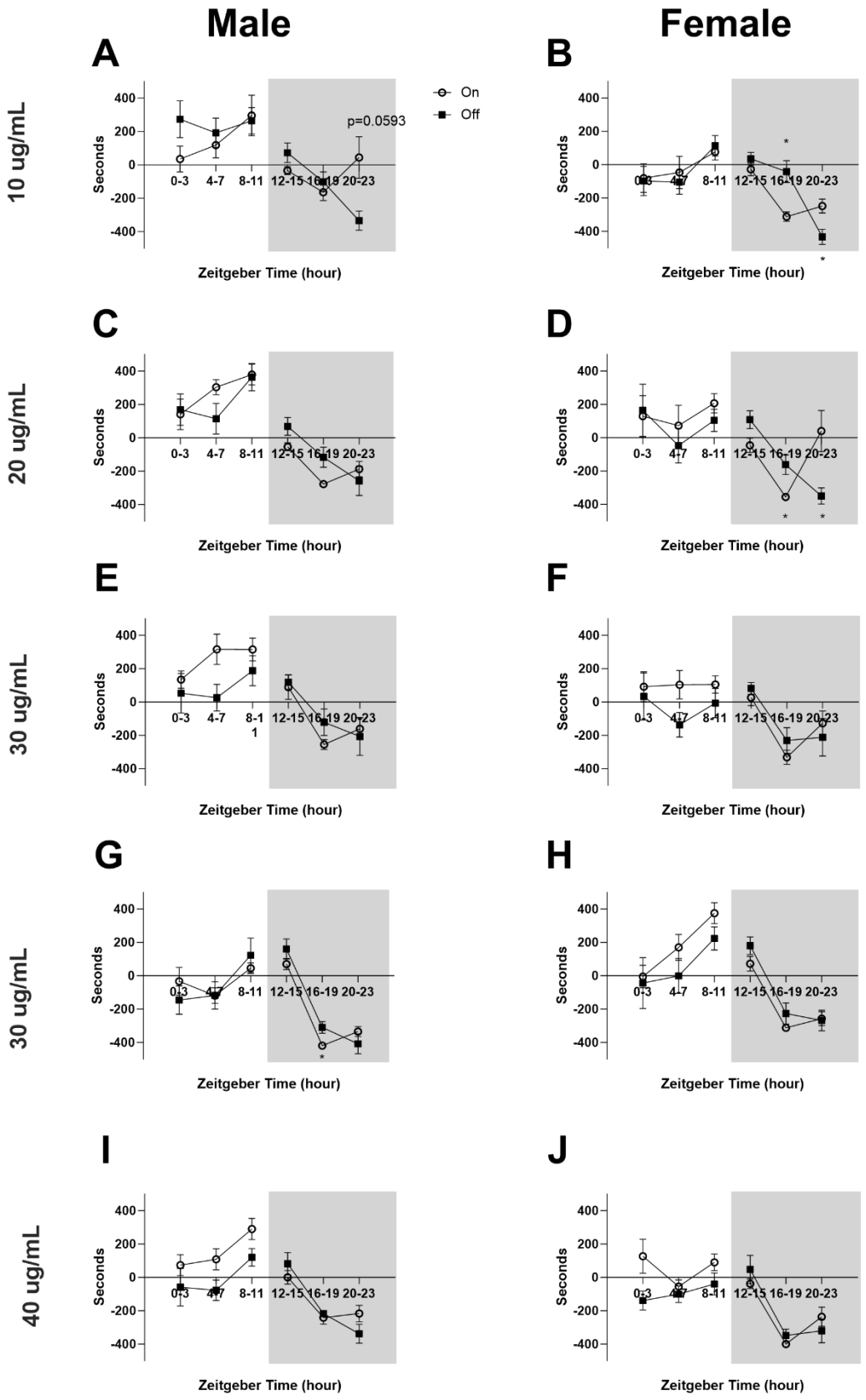
Male
Female
A
B
C
D
E
F
G
H
I
J
10 ug/mL
20 ug/mL
30 ug/mL
30 ug/mL
40 ug/mL
On
Off
p=0.0593
Seconds
400
200
0
-200
-400
0-3
4-7
8-11
12-15
16-19
20-23
Zeitgeber Time (hour)

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): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

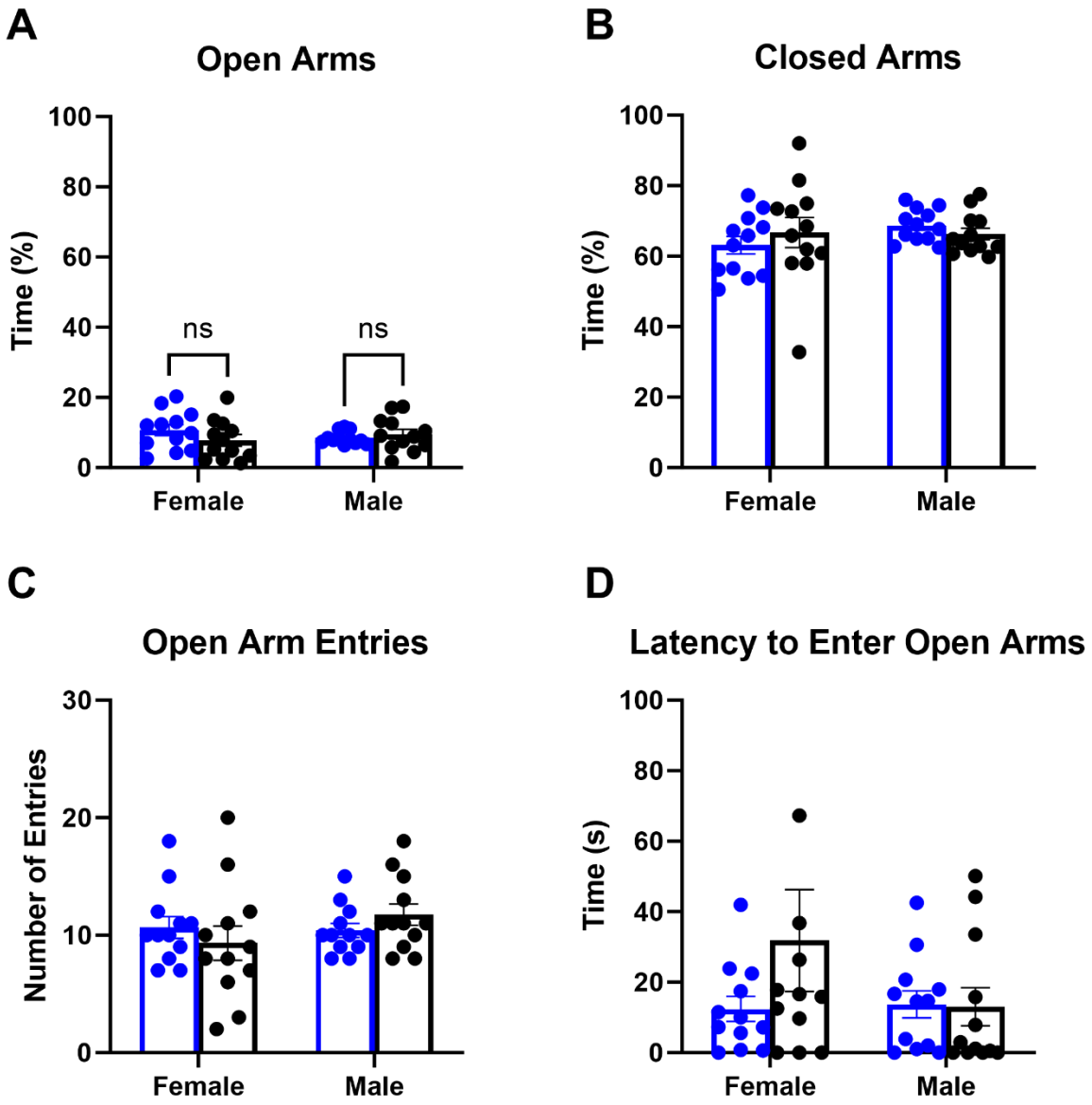

Figure S4. Male and female mice did not show behavioral differences in approach-avoidance conflict through the elevated plus maze assay. **A,B** Both treatment groups of male and female mice did not show difference in the time spent in either the open or closed arms of the maze. **C** Male and female 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