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Probing different paradigms of morphine withdrawal on sleep behavior in male and female C57BL/6J mice

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Abstract

The opioid epidemic has increased dramatically over the last few decades resulting in many suffering from opioid use disorder (OUD). The prevalence of opioids and opioid overdose has been driven by the development of new synthetic opioids, increased availability of prescription opioids, and more recently, the COVID-19 pandemic. As we see increased in exposure to opioids, the United States has also seen increases in the frequency of instances of Narcan (naloxone) administration as a life saving measure for respiratory depression, and, thus, consequently, naloxone-precipitated withdrawal. Sleep dysregulation is one of the main symptoms of OUD and opioid withdrawal syndrome, and therefore should be a key facet of animal models of OUD. Here we examine the effect of precipitated and spontaneous morphine withdrawal on sleep behaviors in C57BL/6J. We find that morphine administration and withdrawal dysregulates sleep, however not equally across morphine exposure paradigms and not qualitatively the same across sexes. Furthermore, many environmental triggers promote relapse to drug seeking/taking behavior, and the stress of disrupted sleep may fall into that category. We find that sleep deprivation dysregulates sleep in mice that had previous opioid withdrawal experience. These data suggest that the 3-day precipitated withdrawal paradigm has the most profound effects on opioid induced sleep dysregulation, and that further validate the construct of the 3-day precipitated withdrawal model as a model for opioid dependence and OUD.

Introduction

Opioid use disorder (OUD) is a chronically relapsing medical condition characterized by cycles of craving, binge, and withdrawal. Exposure to opioids has increased over the last 30 years. In 2015 37.8% of American adults were estimated to have used an opioid in the year prior, and in April 2020 to April 2021 opioid overdose deaths reached a record high of more than 100,000 [1,2]. Opioids can provide profound analgesia, but misuse may result in a physical dependence on opioids, and discontinuation of opioid use may lead to severe withdrawal symptoms known as opioid withdrawal syndrome (OWS). Opioid withdrawal syndrome is characterized by physical and affective symptoms including weight loss, vomiting, aches, insomnia/sleep disturbances, diarrhea, irritability, dysphoria, anxiety, and social deficits [3]. OWS occurs after both spontaneous and precipitated states of withdrawal. Naloxone, a mu opioid receptor antagonist, is a lifesaving medication often used to precipitate withdrawal in humans experiencing respiratory failure, as well as in animal models of OUD[4–8]. As naloxone becomes more available over the counter and through Emergency Medical Services (EMS), we are now seeing increased instances of patients receiving multiple administrations of naloxone, up 26% from 2012-2015 [9]. Recent data indicates that in Guilford County, NC, EMS administration of naloxone is up 57.8% and repeat administration is up 84.8% compared to pre-COVID-19 pandemic numbers [10].

With the increases in opioid use disorder and naloxone administration, it is crucial to better understand the behavioral effects of opioids and naloxone on classic withdrawal symptoms, including sleep disruption. Acute opioid administration, even in non-dependent adults, results in sleep disturbances[11]. Those with a prescription opioid dependency have been shown to differ significantly in several measures of sleep quality compared to those without a dependency on prescription opioids[12]. Far less is known about how sleep is altered during acute opioid exposure and withdrawal in mice. Recent studies have shown that different opioids differentially affect sleep behaviors[13]. Unzaga et al. (2021) found that increasing doses of morphine and fentanyl dose dependently affected sleep and wakefulness in C57 mice[14]. In this study, we examined how three days of repeated morphine exposure and withdrawal can impact sleep behaviors acutely, as well as how it can alter the response to future sleep disruptions. Clinical data indicate that sleep disruptions commonly occur during and following withdrawal from many substances including alcohol, cannabis, and opioids [12,15–18]. Additionally, there is increasing evidence that sleep disruptions may be a biological pressure for relapse, especially in alcohol and opiate withdrawal [17–23] . Some of the effects of opioids on sleep have been recapitulated in preclinical models with cats, rats, and neonatal mice[24–26].

Unfortunately, no studies we have found examined the effect of opioid withdrawal on sleep behavior in female mice. We know little about how opioid withdrawal differentially affects the sexes in rodent models. However, we do know male and female mice experience withdrawal differently, evident in both their behavior responses and the neurobiology [5,27]. Opioid related sleep disturbances can worsen both pain and withdrawal symptoms. Investigating the timeline and nature of these changes, as well as how they might differ between males and females, is critical to better understand and treat opioid withdrawal.

Methods

Subjects

All procedures were approved by the University of North Carolina Institutional Animal Use and Care Committee. Male (N=52) and female(N=52) C57BL/6J mice at least 10 weeks of age were used in all experiments and maintained on a normal 12:12 light–dark cycle, in single housed conditions. All animals had food and water *ad libitum* for the duration of the study. Following the conclusion of data collection, mice were euthanized according to IACUC protocols.

Piezo-Sleep System

Mice were housed individually in PiezoSleep 2.0 (Signal Solutions, Lexington KY) 15.5 cm^2 cages in a room separate from the colony and behavior rooms to help isolate behaviors. Mice were maintained on a 12 hours:12 hours light:dark cycle (for MN vs SN experiments lights were on 7 AM to 7 PM and for the MN vs MS and MS vs SS experiments lights were on 10 am to 10 pm). PiezoSleep is a non-invasive housing and monitoring system that uses piezoelectric pads beneath the cages to detect the mouse's movement. Using the SleepStats software (SleepStats v2.18, Signal Solutions, Lexington KY) vibrational changes are processed to determine sleeping versus waking respiratory patterns, which has been validated using electroencephalography, electromyogram, and visual assessment [28–30].The first two dark cycles were discarded as habituation and baselines consisted of at least 5 days prior to any manipulations.

Withdrawal paradigm

Animals were individually housed in the PiezoSleep chambers while treated for 3 days with the following paradigm, as described previously [27]. Briefly, saline-naloxone (SN) animals were injected with (0.9%) sterile saline (0.1 ml/10g) and 2 hr later with 1 mg/ kg naloxone (in sterile saline 0.1 ml/10g). Morphine-naloxone (MN) animals were injected with 10 mg/kg morphine (in sterile saline 0.1 ml/10 g) and 2 hr later with 1 mg/kg naloxone (in sterile saline 0.1 ml/10 g). Morphine-saline (MS) animals were injected with 10 mg/kg morphine (in sterile saline 0.1 ml/10 g) and 2 hr later with (0.9%) sterile saline (0.1 ml/10g). Saline-saline (SS) animals were injected with (0.9%) sterile saline (0.1 ml/10g) and 2 hr later with (0.9%) sterile saline (0.1 ml/10g). Injections were delivered subcutaneously. The animal numbers and abbreviations for each group and experiment are male morphine naloxone (MMN; N=10) vs. male saline naloxone (MSN; N=10), female morphine naloxone (FMN; N=10) vs. female saline naloxone (FSN; N=10), male morphine naloxone (MMN; N=8) vs. male morphine saline (MMS; N=8), female morphine naloxone (FMN; N=8) vs. female saline naloxone (FSN; N=8), male morphine saline (MMS; N=8) vs. male saline saline (MSS; N=8), and female morphine saline (FMS; N=8) vs. female saline saline (FSS; N=8).

Sleep disruption paradigm

All mice remained in the PiezoSleep chamber following the 3-day withdrawal paradigm. They were left untouched for 5 days and on the 6th day underwent a sleep disruption paradigm. During the beginning of their light cycle (ZT 0-3 for the MN-SN and MS-SS experiments; ZT 1-4 for the MN-MS experiment), the mice were kept awake. This was accomplished by using gentle handling methods (light tapping on cage top, ruffling food in hopper, following mouse in cage with a pencil, removing nesting materials, etc) and cage changes. Mice were then left alone for the remainder of the experiment.

Statistics

All data were analyzed using Graph Pad Prism (version 9.3.1). Data are reported as mean ± SEM. Comparisons were made with either regular or repeated measures 2-way ANOVAS. All post-hoc tests were done using Bonferroni's correction. ANOVAs are reported as (F(DFn,DFd)= F-value, p= P-value).

Results

Modeling morphine withdrawal in PiezoSleep chambers.
We used a three-day withdrawal paradigm previously validated in our lab to assess the effect of morphine withdrawal on sleep behaviors in C57BL/6 [5,27]. This paradigm has been shown to result in sensitization of withdrawal symptoms over the three days of administration[27]. We have also shown various behavioral effects six weeks into forced abstinence[5].In rats, this paradigm has been shown to alter noradrenergic transmission in the ventral bed nucleus of the stria terminalis (vBNST) [31]. Here we evaluate males and females under various conditions including spontaneous withdrawal, precipitated withdrawal, saline control, and naloxone control (**Fig. 1**). Given the previously reported sex differences from our lab, we do not statistically compare the males and females but consider qualitative comparisons. Each experiment consisted of 7 days of habituation and baseline recording, 3 days of withdrawal, 5 days of recovery recordings, a 4-hour sleep disruption study, and additional recovery days at the end (**Fig. 1**).

Significant sex differences in sleep baselines.
We examined baseline days from all animals in the study, females (N=52) and males (N=52) across each zeitgeber hour. Baseline sleep was calculated by averaging each zeitgeber hour across the 3-5 days preceding withdrawal day 1 and calculating the percent of time the mouse spent asleep each hour. We found a main effect of sex (Figure 2A, $F(1,102) = 13.69$, $p=0.0004$), and a significant interaction of sex vs. time (Fig. 2A, $F(1,102)=3.839$, $p<0.0001$). Bonferroni's multiple comparisons showed significant differences in hour 0 ($p<0.0001$), hour 1 ($p<0.0001$), hour 3 ($p=0.0107$), hour 4 ($p=0.0480$), and hour 6 ($p=0.0037$). There were no significant differences during the dark cycle, but males slept significantly more than females during the light cycle (Figure 2B, $F(1,102)=14.57$), $p<0.0001$) and the entire day ($p=0.0027$). We confirmed that there were no baseline differences between treatment groups in each experiment prior to starting any injections (data not shown: FMN vs FSN ($p=0.9275$), FMN vs FMS ($p=0.5275$), FMN vs FSS ($p=0.8731$), MMN vs MSN ($p=0.3955$), MMN vs MMS ($p=0.1607$), and MMN vs MSS ($p=0.6985$).

Effect of morphine withdrawal on sleep in male mice.
On withdrawal day 1, there were no main effects of treatment group across any of the comparisons (MMN vs MSN, MMN vs MMS, and MMS vs MSS) whether graphing the data across 24 hours (3A, 3C, and 3E) or light cycle (3B, 3D, and 3F). There was a main effect of time across all groups, as is expected with diurnal cycles like sleep patterns (Fig 3; (A) MMN vs MSN $F(23,414)=15.23$, $p<0.0001$; (C) MMN vs MMS $F(23,322)=22.34$, $p<0.0001$; (E) MMS vs MSS $F(23,322)=22.34$, $p<0.0001$). There was a significant interaction of group and ZT hour in the MMN vs MSN experiment (Fig 3A; $F(23,414)=6.153$, $p<0.0001$). Bonferroni's multiple comparisons test resulted in the following significant differences: hour 1 ($p=0.0044$), hour 2 ($p<0.0001$), and hour 4($p<0.0001$). Also, in the precipitated withdrawal and naloxone control experiment, post-hoc testing of the light cycle comparisons showed significant differences across 12-hour averages (light vs dark) between the two treatment groups: MSN mice slept more than MMN mice in the light cycle (3B, $p<0.0001$) and significantly less than MMN mice in dark cycle (3B, $p<0.0001$).

Withdrawal day 2 mostly followed the same trends as day 1. All experiments exhibited a main effect of time (Fig 3; (G) MMN vs MSN $F(23,414)=15.59$, $p<0.0001$; (I) MMN vs MMS $F(23,322)=22.3$, $p<0.0001$; (K) MMS vs MSS $F(23,322)=8.809$, $p<0.0001$). The MMN vs MSN experiment had a significant interaction of ZT hour and treatment group (Fig. 3G: $F(23,414) =3.973$, $p<0.0001$) and Bonferroni's post-hoc showed significant differences between precipitated withdrawal and naloxone control at hour 1 ($p<0.0001$), hour 2 ($p=0.064$), hour 4($p=0.0026$), and hour 15 ($p=0.0035$). On WD2, there was also a significant interaction of ZT hour and treatment group for the spontaneous withdrawal vs saline control experiment (3K: $F(23,322)=1.832$, $p=0.0123$) and Bonferroni's post-hoc showed significant differences between MMS and MSS at hour 1 ($p=0.0070$). While assessing the role of treatment groups on sleep during different light cycles, only the MMN vs MSN experiment had a main effect of time (Fig 3H: $F(2,36)=51.7$, $p<0.0001$) and significant interaction of light cycle and treatment group (Fig 3H: $F(2,36)=44.41$, $p<0.0001$). Again, post-hoc analysis showed that MMN mice slept

significantly less in the light (Fig 3H: $p<0.0001$) and significantly more in the dark (Fig 3H: $p<0.0001$) than the MSN mice run at the same time.

On withdrawal day 3, all experiments exhibited a main effect of ZT hour (Fig 3; (M) MMN vs MSN $F(23,414)=13.74$, $p<0.0001$; (O) MMN vs MMS $F(23,322)=17.18$, $p<0.0001$; (Q) MMS vs MSS $F(23,322)=9.681$, $p<0.0001$) as well as a significant interaction of treatment condition vs. time (Fig 3; (M) MMN vs MSN $F(23,414)=4.932$, $p<0.0001$; (O) MMN vs MMS $F(23,322)=3.106$, $p<0.0001$; (Q) MMS vs MSS $F(23,322)=2.453$, $p=0.0003$). Hours 1 ($p<0.0001$) 2 ($p=0.0432$), 4 ($p=0.0184$), 5 ($p=0.0206$), and hour 15 ($p=0.0124$) in the MMN vs MSN experiment was significantly different when examined with post-hoc testing (Fig. 3M). When we examined MMN and MMS conditions (Fig. 3O), hour 3 ($p=0.0002$), hour 5 ($p=0.0018$), hour 21 ($p=0.0211$) and 22 ($p=0.0212$). The only hour with differences between MMS and MSS was hour 1 (Fig. 3Q; $p=0.0002$). When looking at the experiments based on 12 hour light cycles, MMN vs MSN showed a main effect of light cycle (Fig 3N; $F(2,36)=232.5$, $p<0.0001$) and a significant interaction of light cycle and treatment (Fig 3N; $F(2,36)=15.26$, $p<0.0001$) where post-hoc testing showed the MSN mice slept more in the light ($p=0.0004$) and less in the dark ($p=0.0173$) when compared to MMN mice. In the MMS vs MSS experiment, the spontaneous withdrawal group slept signifcantly more than the saline control (Fig. 3R, $p=0.0065$).

Following three days of injections, the mice were left to recover on day 4 (acute withdrawal [27]) without experimental disturbance, and sleep behavior was continuously monitored. There were main effects of ZT hour (Fig 3; (S) MMN vs MSN $F(23,414)=17.45$, $p<0.0001$; (U) MMN vs MMS $F(23,322)=15.21$, $p<0.0001$; (W) MMS vs MSS $F(23,322)=10.55$, $p<0.0001$) and, interestingly, significant interactions of treatment condition and ZT hour (Fig 3; (T) MMN vs MSN $F(23,414)=2.882$, $p<0.0001$; (V) MMN vs MMS $F(23,322)=1.68$, $p=0.0274$; (X) MMS vs MSS $F(23,322)=1.854$, $p=0.0109$). The difference between MMN and MSN was significant at hour 2 ($p=0.0010$) and hour 17 ($p=0.0262$) (Fig. 3S). The MMN mice slept less in the light (Fig. 3T, $p=0.0201$) and more in the dark cycle (Fig. 3T, $p=0.0021$). MMN mice slept more than MMS mice at hour 23 (Fig. 3U, $p=0.0413$). MMS mice slept less than MSS mice at hour 1 (Fig. 3W, $p=0.0255$) and across the entire light cycle (Fig. 3X, $p=0.0081$), but more than the MSS in the dark (Fig. 3X, $p=0.0481$). None of the mice differed across the full day.

Effect of morphine withdrawal on sleep in female mice.

Like what we observed in the male dataset, on withdrawal day 1 FSN, FMN vs FMS, and FMS vs FSS) whether graphing the data across 24 hours (Figures 4A, 4C, and 4E) or light cycle (Figures 4B, 4D, and 4F). There was a main effect of time across all groups (Fig 4; (A) FMN vs FSN $F(23,414) =12.76$, $p<0.0001$; (C) FMN vs FMS $F(23,322) =17.43$, $p<0.0001$; (E) FMS vs FSS $F(23,322) =7.245$, $p<0.0001$). Additionally, there was a significant interaction of group and time in the FMN vs FSN experiment (Fig 4A; $F(23,414) =5.589$, $p<0.0001$). Bonferroni's multiple comparisons test resulted in the following significant differences: hour 2 ($p<0.0001$), hour 4 ($p<0.0001$), hour 5 ($p=0.0038$), hour 17 ($p=0.0058$). Both the FMN vs FSN (Fig. 4B; $F(2,35) =21.09$, $p<0.0001$) and FMS vs FSS (Fig. 4F; $F(2,28) =25.74$, $p<0.0001$) experiments had a main effect of light cycle. The FMN vs FSN experiment was the only comparison exhibiting a significant interaction of light cycle and treatment (Fig. 4B; $F(2,35) =21.13$, $p<0.0001$). FMN slept less in the light cycle (Fig. 4B, $p=0.0021$) and more in the dark cycle ($p=0.0107$).

All experiments exhibited a main effect of time on withdrawal day 2 (Fig 4; (G) FMN vs FSN $F(23,414)=13.95$, $p<0.0001$; (I) FMN vs FMS $F(23,322)=16.29$, $p<0.0001$; (K) FMS vs FSS $F(23,322)=8.058$, $p<0.0001$). Unlike the male sleep results, all three experiments had significant interactions of time and treatment group on day 2. The FMN vs FSN experiment had a significant interaction of treatment group and time (Fig. 4G: $F(23,414) =3.488$, $p<0.0001$) and Bonferroni's post-hoc showed significant differences between precipitated withdrawal and naloxone control at hour 1 ($p=0.0017$) and hour 4 ($p=0.0002$). The precipitated versus spontaneous withdrawal experiment had similar differences in interactions (Fig. 4I: $F(23,322) =3.106$, $p<0.0001$) with differences at hour 3 ($p=0.0248$), hour 5 ($p=0.0192$), and hour 6 ($p<0.0001$). There was also a significant

interaction of ZT hour and treatment group for the spontaneous withdrawal vs saline control experiment (Fig. 4K: $F(23,322) = 1.618$, $p=0.0380$). While assessing the role of treatment groups on sleep during different light cycles, only the FMN vs FSN experiment had a main effect of time (Fig. 4H: $F(2,36) = 15.00$, $p<0.0001$) and significant interaction of light cycle and treatment group (Fig 4H: $F(2,36) = 14.79$, $p<0.0001$). Post-hoc analysis showed that FMN mice slept significantly less in the light (Fig 4H: $p=0.0128$) and more in the dark ($p=0.0062$) than the FSN mice run at the same time.

As with the males on withdrawal day 3, all experiments exhibited a main effect of ZT hour (Fig 4; (M) FMN vs FSN $F(23,414) = 9.122$, $p<0.0001$; (O) FMN vs FMS $F(23,322) = 16.71$, $p<0.0001$; (Q) FMS vs FSS $F(23,322)=9.575$, $p<0.0001$) as well as a significant interaction of treatment condition and ZT hour (Fig 4; (M) FMN vs FSN $F(23,414) = 3.186$, $p<0.0001$; (O) FMN vs FMS $F(23,322) = 2.206$, $p=0.0014$; (Q) FMS vs FSS $F(23,322) = 1.724$, $p=0.0220$). Hour 1 in the FMN vs FSN experiment was significantly different with post-hoc testing (Fig 4M; $p<0.0001$). When we examined FMN and FMS conditions (Fig. 4O) only hour 5 was significant ($p=0.0018$). Between FMS and FSS, the FMS animals slept less ($p=0.0195$) in the light and more in the dark ($p=0.0069$) than the FSS animals (Fig. 4Q). FMN vs FSN showed a main effect of light cycle (Fig 4N; $F(2,35) = 76.47$, $p<0.0001$) and a significant interaction of light cycle and treatment (Fig 4N; $F(2,35) = 6.260$, $p<0.0001$) where post-hoc testing showed the FMN mice slept more in the dark ($p=0.0158$) compared to FSN mice.

On day 4, there were main effects on ZT hour for all groups (Fig 4; (S) FMN vs SN $F(23,414)=9.534$, $p<0.0001$; (U) FMN vs FMS $F(23,322) = 9.426$, $p<0.0001$; (W) FMS vs FSS $F(23,322) = 7.590$, $p<0.0001$) and significant interactions of treatment condition and ZT hour (Fig 4; (T) FMN vs FSN $F(23,414) = 1.684$, $p=0.0257$; (V) FMN vs FMS $F(23,322)=1.631$, $p=0.0355$; (X) FMS vs FSS $F(23,322) = 2.474$, $p=0.0003$). Post-hoc analysis showed FMN mice slept more than FSN mice at hour 17 ($p=0.0482$) and FMS mice slept more than FSS mice at hour 17 ($p=0.0020$). FMS mice slept more than FSS mice at hour 3 ($p=0.0121$) and at hour 17($p= 0.0037$; Fig. 4W).

Male sleep displaced from baseline following withdrawal.

To investigate how individual animals shifted their sleep patterns during the treatment and withdrawal from opioids, we also examined the data as sleep displaced from baseline. All experiments are shown as cumulative minutes displaced from baseline across a 24-hour period. Percentages were converted to total minutes spent asleep and then subtracted from the total minutes slept up to that point during baseline for each individual animal. On withdrawal day 1, no experiments showed main effects of treatment group between males. There was a significant interaction of cumulative time and group in the MMN vs MSN experiment (Fig.5A $F(23,414) = 4.909$, $p<0.0001$). On withdrawal day 2, only MMN vs MSN had a main effect of group (Fig. 5D $F(1,18) = 4.572$, $p=0.0465$) and all three experiments had significant interactions (Fig.5 (D) MMN vs MSN $F(23,414) = 2.018$, $p=0.0039$; (E) MMN vs MMS $F(23,322) = 1.786$, $p=0.0157$; (F) MMS vs MSS $F(23,322)=3.536$, $p<0.0001$). On withdrawal day 3, only MMN vs MSN had a main effect of group (Fig. 5G $F(1,18) = 5.462$, $p=0.0312$) and a significant interaction ($F(23,414) = 2.938$, $p<0.0001$), with a difference at hour 6 ($p=0.0317$). The MMS vs MSS experiment also showed a significant interaction (Fig. 5I $F(23,322) = 2.421$, $p=0.0004$) where hour 4 showed significant differences ($p=0.0468$). On the recovery day, MMN vs MSN showed a main effect of group (Fig.5J $F(1,18) = 5.109$, $p=0.0364$) and a significant interaction ($F(23,414) = 1.931$, $p=0.0065$). MMN vs MMS also showed a main effect (Fig. 5K $F(1,14) = 5.252$, $p=0.0379$), but no significant interaction. Finally, MMS vs MSS showed a significant interaction (Fig.5L $F(23,322)=3.048$, $p<0.0001$).

Female sleep displaced from baseline following withdrawal.

In the female mice, there were no significant differences in how each group differed from their baseline on withdrawal day 1. FMN vs FSN had a significant interaction (Fig. 6A $F(23,414) = 3.908$, $p<0.0001$). On WD2, the FMN vs FMS experiment had a significant interaction (Fig. 6E $F(23,322) = 1.645$, $p=0.0332$). On WD3, there was a main effect of group between FMN and FSN (Fig.6G $F(1,18) = 4.573$, $p=0.0464$). There was also a significant interaction in the FMS vs FSS experiment (Fig. 6I, $F(23,322) = 1.967$, $p=0.0057$), which persisted to

the recovery day (Fig. 6L, $F(23,322) = 37.01$, $p<0.0001$). There were no other differences that lasted into the recovery days for females.

Sleep disruption in male and female mice.

Following 6 days of forced abstinence where the mice were not manipulated by the investigators, we next conducted sleep disturbance assays. All mice were kept awake for 4 hours at the beginning of their light cycle, staring at either ZT0 or ZT1 (see methods). All male groups had significant main effects of time (Fig 7; (A) MMN vs MSN $F(23,414) = 38.79$, $p<0.0001$; (C) MMN vs MMS $F(23,322) = 29.39$, $p<0.0001$; (E) MMS vs MSS $F(23, 322)=29.41$, $p<0.0001$). The precipitated withdrawal group had a significant interaction (Fig. 7C, $p=0.0242$) with a difference at ZT 17 ($p=0.0035$). One day following this sleep disruption, MMN and MSN responded differently as noted by a significant interaction of group and time (Fig. 7G $F(23,414) = 1.950$, $p=0.0058$). Hour 2 ($p=0.0422$) and hour 16 ($p=0.0166$) were both significant during post-hoc testing, but in opposite directions (Fig. 7G). MMN vs MMS also had a significant interaction (Fig. 7I $F(23,322)=1.913$, $p=0.0078$) with the difference occurring at hour 0 ($p=0.011$). There were no significant differences between MMS and MSS. For the females, only FMN vs FSN had a significant interaction on the sleep disruption day (Fig. 8A $F(23,414) = 1.606$, $p= 0.0388$) and differences at hour 20 ($p= 0.0264$) and hour 21 ($p=0.0043$). While there was no interaction in the FMN vs FMS experiment, there was a significant difference at hour 20 ($p=0.0047$). On the recovery day, FMN vs FMS had a main effect of treatment group (Fig. 8I, $F(1,14) = 5.840$, $p=0.0299$) and difference at hour 20 ($p=0.0154$).

Cumulative minutes displaced from baseline sleep during sleep disruption and recovery.

All male groups differed similarly from their baselines and there were no significant differences between groups on the sleep disruption and recovery days (Fig. 9). Female precipitated withdrawal animals had significantly different sleep on the sleep disruption day (Fig 10B, $F(1,14)=4.721$, $p=0.0474$) as well as a significant interaction of hour and group ($F(23,322)=1.742$, $p=0.0199$). By hour 9, the FMS group had slept more than 100 minutes less than their sleep up to hour 9 on their baseline. On the following day, the FMN vs FMS experiment was trending towards being significantly different (Fig. 10E, $p=0.0731$).

Discussion

Sleep is governed by many brain regions and neurotransmitters. EEG studies have identified the various regions involved in each stage of sleep including portions of cortex, thalamus, hypothalamus, hippocampus, basal forebrain and others [32]. As people experience stressors or sleep disruptions, the brain regions involved in sleep and the connectivity between them is altered as the brain tries to maintain a homeostasis. In particular following sleep disruption or restriction, the amygdala becomes hyperactive and more responsive to negative stimuli [33,34]. Sleep deprivation also results in decreased pain tolerance which has been tied to the nucleus accumbens, thalamus, and insula [35].

There are many brain regions known to be involved in sleep, that are also implicated in addiction/reward circuitry. In particular, monoaminergic nuclei fill these niches. The locus coeruleus (LC) projects to the basal forebrain, bringing dense noradrenergic inputs that are known to correlate with arousal states. The LC is the beginning of a wake-promoting circuit that using monoaminergic signaling through the midbrain and to the frontal cortex, and receives feedback from orexinergic neurons in the lateral hypothalamus [36]. Activation of the LC promotes arousal, but inactivation of the LC results in increases in NREM sleep [36]. During opioid withdrawal, the LC neurons are activated and there is an increase in norepinephrine in the ventral forebrain drives opioid withdrawal-induced aversion [37]. There is also an increase in norepinephrine in the ventral bed nucleus of the stria terminalis (vBNST) following opioid exposure and withdrawal, although the source of this NE is mainly from the medullary noradrenergic neurons [31]. In both our male and female mice, we see an increase in arousal following morphine administration, potentially due to DA activation. The dorsal raphe nucleus (DRN), a largely serotonergic and dopaminergic nucleus, projects to the ventral tegmental area (VTA), prefrontal cortex (PFC), amygdala, basal ganglia, and lateral habenula. For example, the VTA is considered the preeminent common substrate for all rewarding substances and behaviors. It sends dopamine to the nucleus accumbens and is highly implicated in facilitating rewarding behaviors and self-administration of drugs. The VTA consists of both dopaminergic and non-dopaminergic (GABAergic) neurons, each which have been shown to play a role in control of sleep behaviors [38,39]. The GABAergic neurons in the VTA show increased firing during REM sleep, and decreased firing during waking behaviors [38]. These neurons synapse onto other GABA-neurons as well as onto the DA neurons in the VTA [40]. Inhibition of these dopamine neurons results in sleep induction and sleep preparation behaviors such as nest-building [39].

Not only do sleep and drug exposure/withdrawal share aspects of their neural circuitry, but they are also both very time-sensitive experiences for humans and animals. For example, sleep occurs on a diurnal cycle with various stages comprised of different waveforms while opioid withdrawal occurs in stages (acute to protracted).

Here we examined how different paradigms of opioid withdrawal (and their respective controls) modulate sleep behavior in male and female mice using a non-invasive sleep measurement across multiple days, and during a 4-hour sleep disturbance and recovery. We compare a 3-day precipitated morphine withdrawal paradigm that we and others have used to model the development of opioid dependence/tolerance as compared to spontaneous withdrawal from the same dose of morphine. We also compare these paradigms to their respective controls. Our data suggests that repeated withdrawal dysregulates sleep behavior, and the precipitated withdrawal paradigm results in greater sleep dysregulation, including subsequent drug free recovery days and alterations in sleep drive following sleep disturbance. Additionally, we observed differences in some of these metrics between male and female subjects.

Difference in baseline sleep behaviors in male and female mice. In this study, we compared our model of OUD – a 3-day precipitated morphine withdrawal paradigm which results in acute withdrawal responses (ie. sensitization of escape jumps, paw tremors, and fecal boli over 3 days[5]) as well as protracted withdrawal responses (ie. changes in open field behaviors, social interaction, and increased locomotion[5]) -- to spontaneous morphine withdrawal, in the context of sleep dysregulation. Our previous studies also began to tease apart the sex differences that occur during acute and protracted withdrawal. We conducted these paradigms inside our PiezoSleep chambers and examined the effects of morphine withdrawal on the sleep behaviors of C57CL/6J male and female mice (**Fig. 1**). We saw that male and female C57BL/6J mice sleep

differently at baseline, with the males sleeping on average 58.3% of the light cycle and 22.9% of the dark cycle while females slept 52.0% of the light cycle and 22.6% of the dark cycle on average (**Fig. 2**). The pattern in sleep differences between males and females are consistent with those seen in healthy adult humans [41]. Once we established the baseline sleep behaviors, the mice were then exposed to morphine and withdrawal. Our previous studies indicate that experiences during the acute withdrawal may have both immediate and long lasting effects [22,23]. Due to the significant difference between male and female baselines, the sexes are only compared qualitatively for the remainder of the discussion.

Validation of effects during precipitated withdrawal paradigm. We showed that our previously published naloxone-precipitated morphine withdrawal paradigm results in sleep disruptions similar to observations in clinical reports [12,17,23]. Morphine results in decreased sleep in the light cycle and increased sleep in the dark cycle for both males and females across all three days of injections (**Fig. 3 & 4**). This parallels the human experience in which those in taking opioids or withdrawn from opioids report increased daytime sleepiness followed by insomnia at night [43–46]. Naloxone, on the other hand, results in an immediate increase of sleep following injection in either male or female animals (**Fig.3 A, B; Fig.4 A, B**). In non-opioid-dependent people, naloxone alone results in increased latency to reach REM sleep, duration of REM, and number of REM cycles [42] which is likely due to activation of the LC, which only ceases firing during REM sleep. In those with an opioid use disorder, maintenance treatment of buprenorphine and naloxone (Suboxone) results in improved sleep compared to those going through treatment without the combination therapy, but the sleep patterns do not return to baseline [48–50]. Across all our mice, the total time spent sleeping was not different between groups on any given day. This suggests that sleep that was displaced by morphine exposure and withdrawal was gained at other points during the day.

Sleep disruptions continue into the first day of abstinence in both male and female mice.
While the 3-day precipitated withdrawal paradigm was originally developed to promote the rapid development of opioid dependence, the current opioid epidemic has resulted in people receiving doses of Narcan (naloxone) to alleviate respiratory depression and save lives. Moreover, the rates of people receiving multiple doses of Narcan from Emergency Medical Services is additionally on the rise (up 26% from 2012-2015 [9]), The increasing frequency of repeated withdrawals and repeated dosing of naloxone begs the question of what rapid withdrawal itself, not merely opioid exposure and spontaneous withdrawal, does to the brain. Here, we see that there are acute effects of morphine withdrawal on sleep that persist into the day following the last exposure and withdrawal. Both males and females show interactions between the hour and treatment group, across all experiments (**Fig. 3,4: S,U,W**). Male morphine-naloxone mice showed a significant reduction in sleep during the light phase (similar to the time point where they received morphine/naloxone on the preceding days), as well as an increase in sleep during the dark when compared to their saline-naloxone controls (**Fig. 3T**). This is not the case, however, for the females (**Fig. 4T**). The persistence of disruptions into the 24-36 hours after the last dose of morphine is comparable to that seen in the human condition, where studies have shown disruptions may never completely disappear which may be due to external stressors (see discussion on sleep disturbance below) [49,50]. Interestingly, at this same 24-hour time point, our lab has shown that GABAergic signaling in the BNST is altered following opioid withdrawal [27]. Intriguingly, the plasticity was different in males and females: Males exhibited increased frequency of spontaneous IPSCs, while the females showed decreased frequency of sIPSCs. An increase in GABAergic signaling in the BNST might result in a disinhibition of VTA GABA neurons, which have been shown to have decreased activity during awake states [27,38]. This circuit provides an avenue for explanation given the differences in GABA signaling and sleep behaviors in acute withdrawal. We have also examined various behaviors 6 weeks following the precipitated withdrawal and seen several sex differences [5]. This indicated that in the future, it might be beneficial to observe sleep behavior at a more protracted time point similar to our previous studies of protracted withdrawal behavior [5].

Within-animal comparisons elucidate differential responses by male and female mice.
In addition to assessing the sleep percentage by hour, we have assessed the sleep by calculating cumulative difference in minutes of sleep obtained throughout the day. This form of analysis considers within-subject

variability and allows each mouse to serve as their own baseline. Here, we can more clearly visualize the effect of naloxone in combination with morphine on male mice (**Fig. 5K**). On the recovery day, males, but not females, exhibited a significant main effect of group between the precipitated and spontaneous withdrawal group. This is particularly interesting given that the precipitated and spontaneous withdrawal groups behaved almost identically to each other on the three days of withdrawal, with only a significant interaction of hour and group on WD2 (**Fig. 5 E**). However, during acute withdrawal, the precipitated withdrawal animals slept significantly less than the spontaneous withdrawal, indicating a role of withdrawal severity on symptoms (Fig. 5K). For the females, the spontaneous and precipitated withdrawal groups had a significant interaction but no other effects (**Fig. 6E**). These findings indicate that males are potentially more affected by the combination of morphine and naloxone than female mice, specifically at the dose of 1mg/kg of naloxone. One clinical study showed that women are more likely than men to use opioids while on a buprenorphine/naloxone treatment [51], and another concluded that women and men respond differently to naloxone depending on the dose [52].

Our main finding is that male mice are more sensitive to morphine withdrawal induced sleep disturbances than female mice.

Sleep disruptions one week after withdrawal result in changes in sleep on recovery day in males and females. Examining sleep following a sleep disturbance allows for the analysis of how our manipulations alter the homeostatic sleep drive. Sleep disturbance, however, is also a stressor that many of us experience in our daily lives. Therefore, we wanted to observe how a 4-hour sleep deprivation early in the dark cycle would alter future sleep behavior. We performed these experiments 6 days after the final drug treatments because all mice returned to regular sleep cycles by day 3 of recovery (data not shown). Here again we observed interesting qualitative differences between the sexes. Examining the precipitated withdrawal vs. naloxone control group, on the day of sleep deprivation, there were no differences between the male mice, however male mice exhibit significant interactions and timepoint differences on the day following sleep disruptions **(Fig 7B).** Again, we observed enhanced waking behavior at hour 2 and enhanced sleep at hour 16, mimicking what was observed on recovery day 1. Female mice, in contrast have a significant treatment by hour interaction on the day of the sleep disturbance, and mice that had previously experienced precipitated withdrawal had enhanced active-period sleepiness in hours 20 and 21 **(Fig 8A**) but did not show significant differences the following day. Together, these changes might indicate that stressors occurring during abstinence impact male and female mice differently. We know that stress is often a driving force behind relapse to drug taking behavior and preclinical data implicates noradrenergic signaling in the extended amygdala in reinstatement [42,53,54]. Sleep, stress, and opioid signaling overlap in these extended amygdala circuits, and all utilize noradrenergic signaling. Additional studies are needed to determine the impact of these specific circuits on sleep behavior in withdrawal, but they present a space for interesting sex differences.

Limitations. Sleep patterns are very specific to individuals due to the drive of social and environmental pressures in addition to the genetic and biological drivers. As such, we see a lot of variability between subjects. This can often make it hard to identify the real effects of treatments. Not only can there be a lot of between subject variability, but there can also be a lot of variability due to environmental changes. For example, the MS vs. SS experiment varies from the other experiments run in this study. We believe this was due to vivarium variability and this underscores the importance of conducting simultaneous controls. We feel confident in the effects seen because the results were consistent across all animals in two cohorts. Additionally, many sleep studies use electroencephalography (EEG) to measure brain activity and determine sleep. Here we use a non-invasive PiezoSleep chamber to measure mouse activity. The PiezoSleep chambers have been validated thoroughly and compared to other methods used in the sleep field [28,30,55].

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MLB conceptualized experiments, conducted experiments, analyzed data, and wrote manuscript. JSL conducted experiments and analyzed data. IMB, AT, and PJP conducted experiments. LT and GD advised on experiments and provided feedback on the manuscript. ZAM conceptualized experiments, analyzed data, and wrote manuscript.

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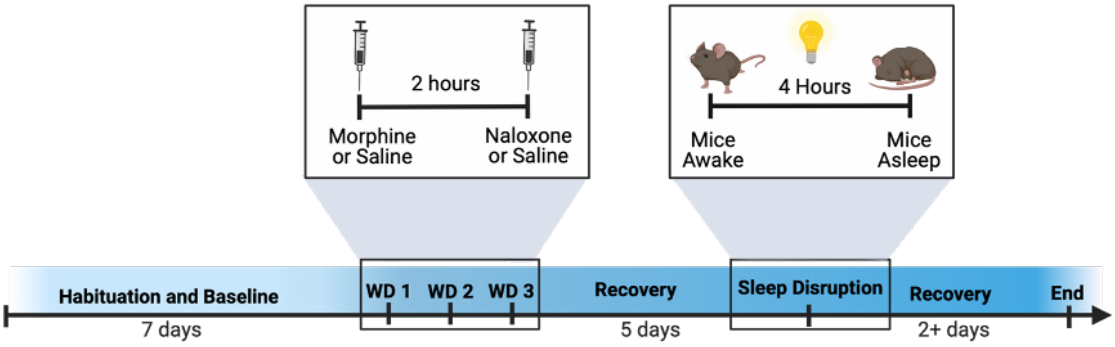

Abbreviation	Sex	Injection 1	Injection 2	Group
FMN/MMN	Female/Male	Morphine	Naloxone	Precipitated WD
FMS/MMS	Female/Male	Morphine	Saline	Spontaneous WD
FSN/MSN	Female/Male	Saline	Naloxone	Naloxone Control
FSS/MSS	Female/Male	Saline	Saline	Saline Control

Figure 1. ***Precipitated-withdrawal paradigm in PiezoSleep chambers.*** Experimental timeline including habituation, baseline, morphine exposure and withdrawal, observation, and sleep disruption. Table detailing the eight different treatment groups (four per sex).

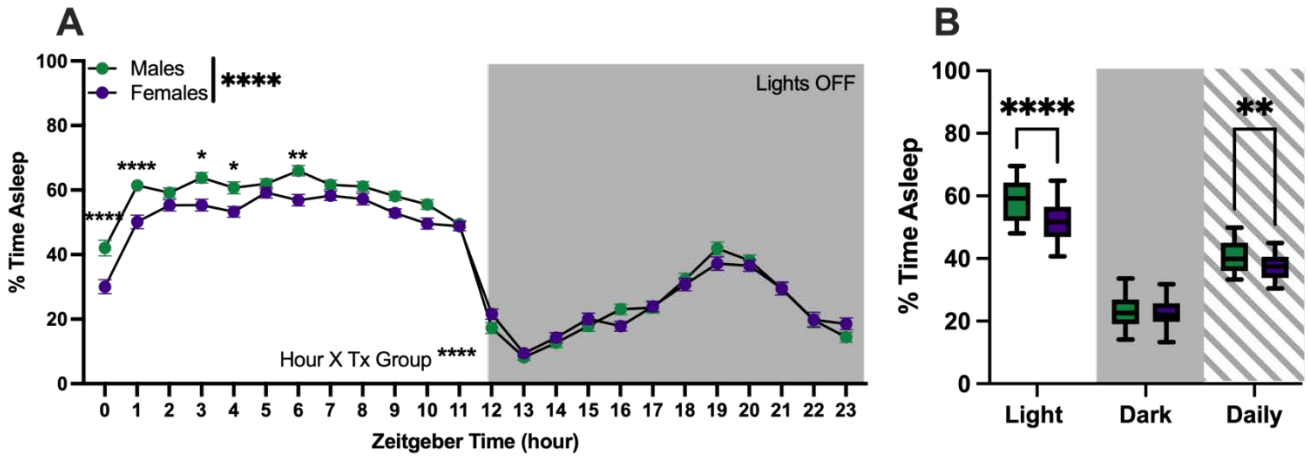

Figure 2. ***Sex differences in baseline sleep behaviors of C57 mice.*** **(A)** Average of all baseline days shown as percent time spent sleeping of each hour in a 24-hour period, shown as Zeitgeber Time (ZT). ZT0 is the start of the light cycle and ZT12 is the start of the dark cycle. **(B)** Percent time spent sleeping in a 12-hour period of all baseline days (light or dark) and across all 24-hours of all baseline days.

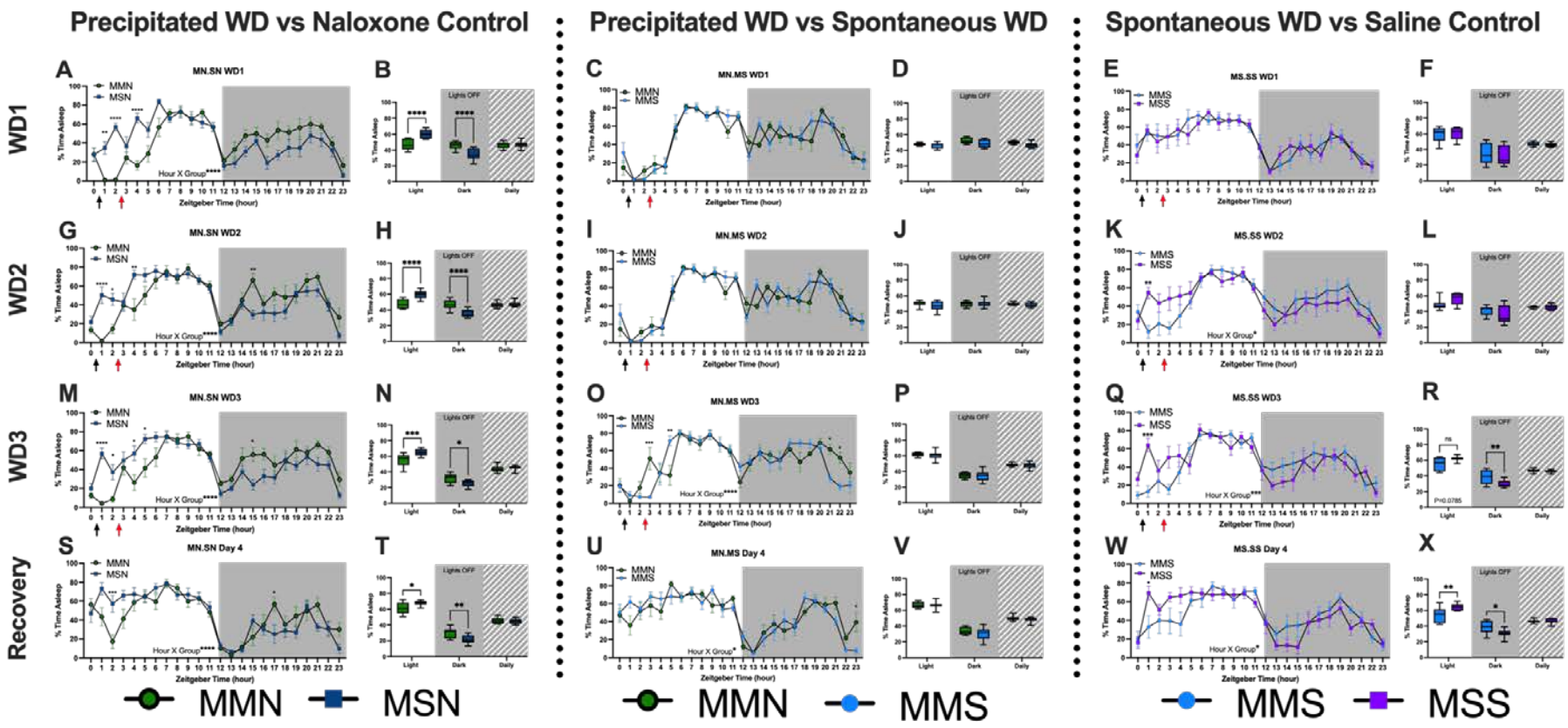
Precipitated WD vs Naloxone Control
Precipitated WD vs Spontaneous WD
Spontaneous WD vs Saline Control
WD1
WD2
WD3
Recovery
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
MN.SN WD1
MN.MS WD1
MS.SS WD1
MN.SN WD2
MN.MS WD2
MS.SS WD2
MN.SN WD3
MN.MS WD3
MS.SS WD3
MN.SN Day 4
MN.MS Day 4
MS.SS Day 4
MMN
MSN
MMS
MSS
% Time Asleep
Zeitgeber Time (hour)
Hour X Group
Lights OFF
Light
Dark
Daily
MMN
MSN
MMN
MMS
MMS
MSS

Figure 3. ***Sleep behavior of male mice during morphine exposure and withdrawal.*** *Graphs depict either 24-hour sleep trace with percent sleep per hour, percent sleep per light cycle, or daily percent sleep. Each row is a different day (withdrawal days 1-3 and then recovery day 1). Black arrows indicate morphine or saline injections (injection #1) and red arrows indicate saline or naloxone injections (injection #2). (A) withdrawal day 1 MN vs SN 24-hour trace (B) withdrawal day 1 MN vs SN by light cycle (C) withdrawal day 1 MN vs MS 24-hour trace (D) withdrawal day 1 MN vs MS by light cycle (E) withdrawal day 1 MS vs SS 24 hour trace (F) withdrawal day 1 MS vs SS by light cycle (G) withdrawal day 2 MN vs SN 24-hour trace (H) withdrawal day 2 MN vs SN by light cycle (I) withdrawal day 2 MN vs MS 24-hour trace (J) withdrawal day 2 MN vs MS by light cycle (K) withdrawal day 2 MS vs SS 24 hour trace (L) withdrawal day 2 MS vs SS by light cycle (M) withdrawal day 3 MN vs SN 24-hour trace (N) withdrawal day 3 MN vs SN by light cycle (O) withdrawal day 3 MN vs MS 24-hour trace (P) withdrawal day 3 MN vs MS by light cycle (Q) withdrawal day 3 MS vs SS 24 hour trace (R) withdrawal day 3 MS vs SS by light cycle (S) recovery day MN vs SN 24-hour trace (T) recovery day MN vs SN by light cycle (U) recovery day MN vs MS 24-hour trace (V) recovery day MN vs MS by light cycle (W) recovery day MS vs SS 24 hour trace (X) recovery day MS vs SS by light cycle. Each point or bar represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

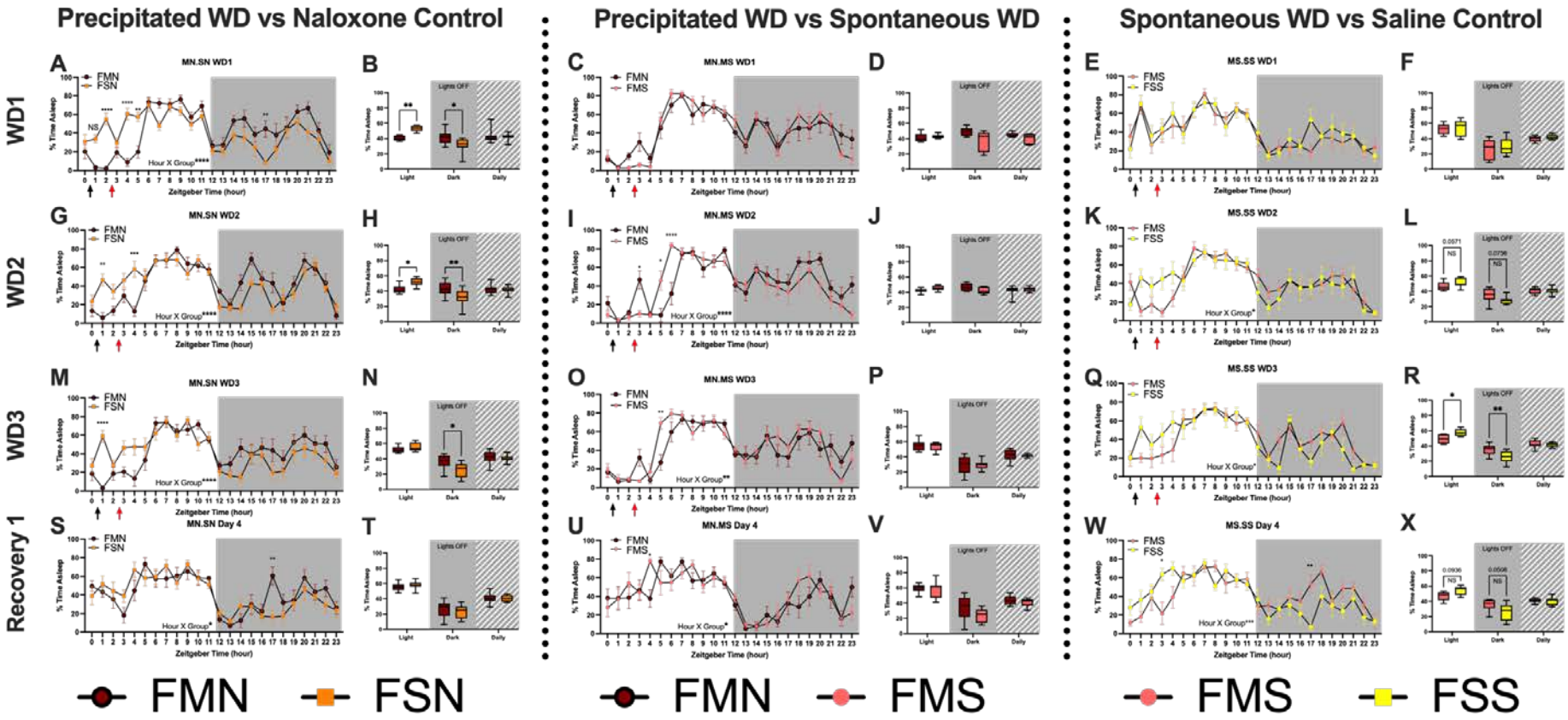
Precipitated WD vs Naloxone Control
Precipitated WD vs Spontaneous WD
Spontaneous WD vs Saline Control
WD1
WD2
WD3
Recovery 1
A
MN.SN WD1
B
C
MN.MS WD1
D
E
MS.SS WD1
F
G
MN.SN WD2
H
I
MN.MS WD2
J
K
MS.SS WD2
L
M
MN.SN WD3
N
O
MN.MS WD3
P
Q
MS.SS WD3
R
S
MN.SN Day 4
T
U
MN.MS Day 4
V
W
MS.SS Day 4
X
FMN
FSN
FMS
FSS
% Time Asleep
Zeitgeber Time (hour)
Hour X Group
Lights OFF
Light
Dark
Daily
FMN
FSN
FMN
FMS
FMS
FSS

Figure 4. ***Sleep behavior of female mice during morphine exposure and withdrawal.***

*Graphs depict either 24-hour sleep trace with percent sleep per hour, percent sleep per light cycle, or daily percent sleep. Each row is a different day (withdrawal days 1-3 and then recovery day 1). Black arrows indicate morphine or saline injections (injection #1) and red arrows indicate saline or naloxone injections (injection #2). (A) withdrawal day 1 MN vs SN 24-hour trace (B) withdrawal day 1 MN vs SN by light cycle (C) withdrawal day 1 MN vs MS 24-hour trace (D) withdrawal day 1 MN vs MS by light cycle (E) withdrawal day 1 MS vs SS 24 hour trace (F) withdrawal day 1 MS vs SS by light cycle (G) withdrawal day 2 MN vs SN 24-hour trace (H) withdrawal day 2 MN vs SN by light cycle (I) withdrawal day 2 MN vs MS 24-hour trace (J) withdrawal day 2 MN vs MS by light cycle (K) withdrawal day 2 MS vs SS 24 hour trace (L) withdrawal day 2 MS vs SS by light cycle (M) withdrawal day 3 MN vs SN 24-hour trace (N) withdrawal day 3 MN vs SN by light cycle (O) withdrawal day 3 MN vs MS 24-hour trace (P) withdrawal day 3 MN vs MS by light cycle (Q) withdrawal day 3 MS vs SS 24 hour trace (R) withdrawal day 3 MS vs SS by light cycle (S) recovery day MN vs SN 24-hour trace (T) recovery day MN vs SN by light cycle (U) recovery day MN vs MS 24-hour trace (V) recovery day MN vs MS by light cycle (W) recovery day MS vs SS 24 hour trace (X) recovery day MS vs SS by light cycle. Each point or bar represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

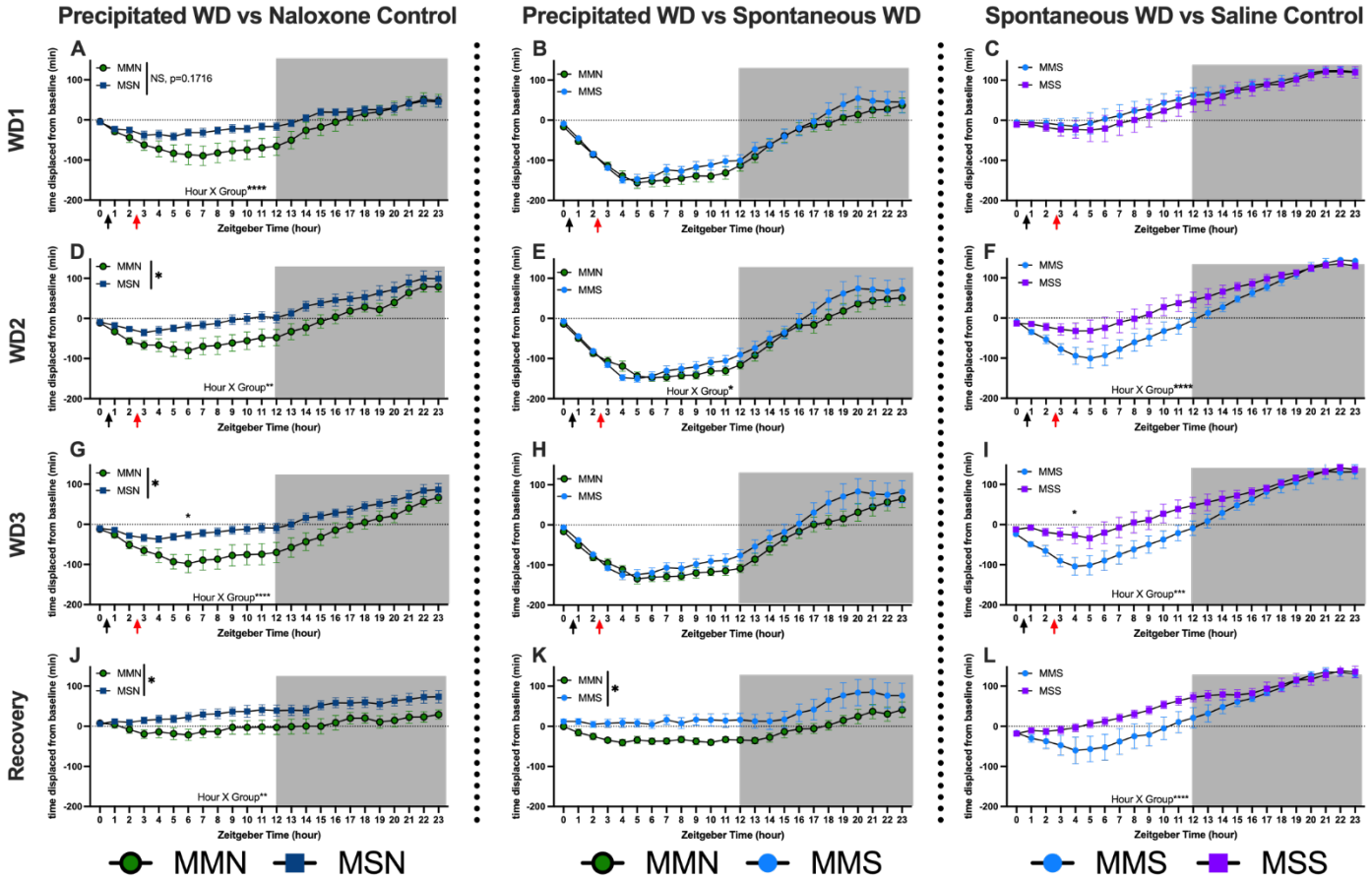

Figure 5. ***Cumulative difference in minutes of sleep of male mice during morphine exposure and withdrawal.*** Minutes of sleep compared to baseline sleep. Graph shows minutes of sleep subtracted from baseline minutes, cumulative by hour. Black arrows indicate morphine or saline injections (injection #1) and red arrows indicate saline or naloxone injections (injection #2). Each row is a different day (withdrawal days 1-3 and then recovery day 1). Every column is a different experiment (MMN vs MSN, MMN vs MMS, and MMS vs MSS). Grey box shows lights off. Each point represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

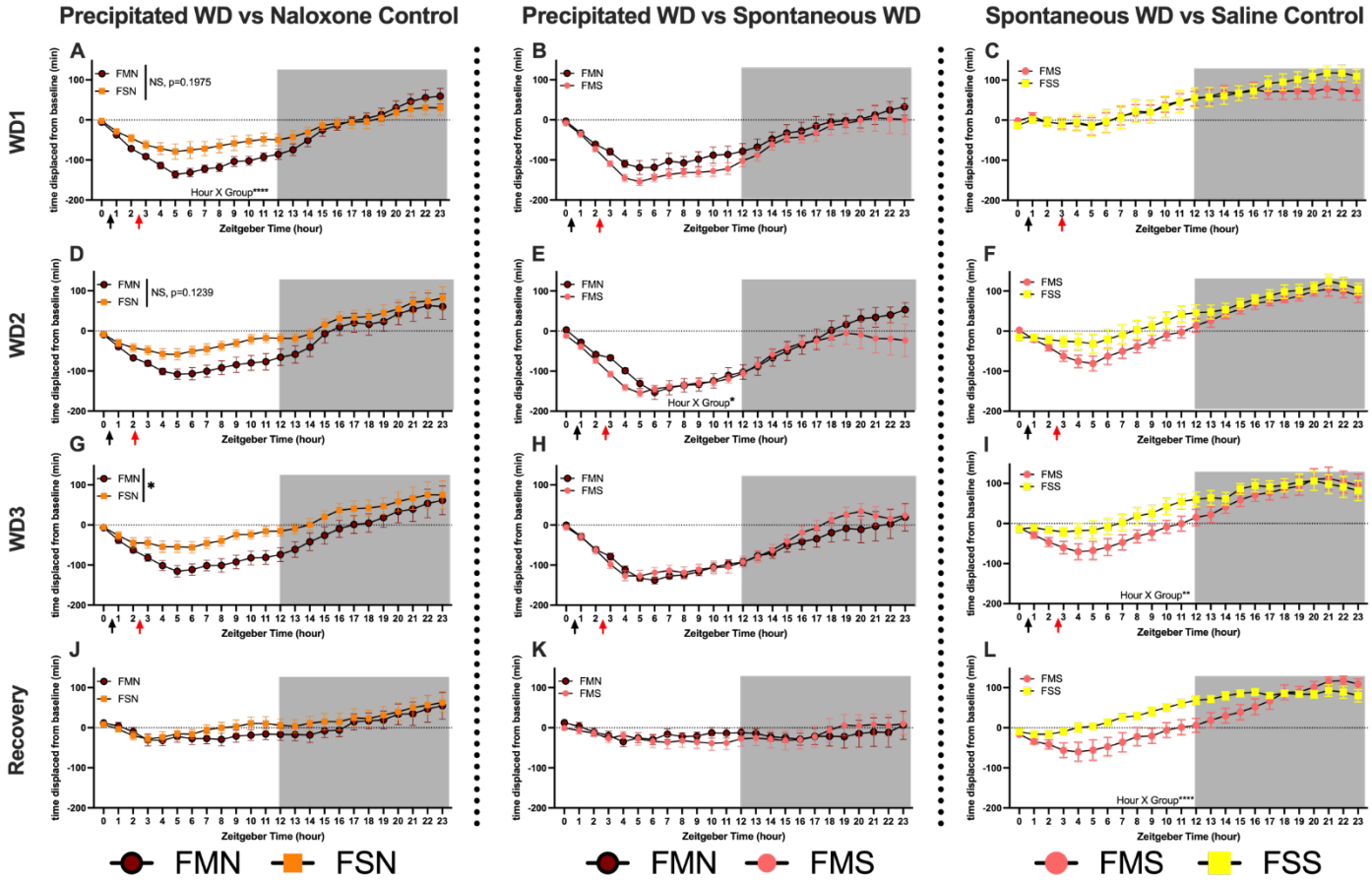

Figure 6. ***Cumulative difference in minutes of sleep of female mice during morphine exposure and withdrawal.*** Minutes of sleep compared to baseline sleep. Graph shows minutes of sleep subtracted from baseline minutes, cumulative by hour. Black arrows indicate morphine or saline injections (injection #1), and red arrows indicate saline or naloxone injections (injection #2). Each row is a different day (withdrawal days 1-3 and then recovery day 1). Every column is a different experiment (FMN vs FSN, FMN vs FMS, and FMS vs FSS). Grey box shows lights off. Each point represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

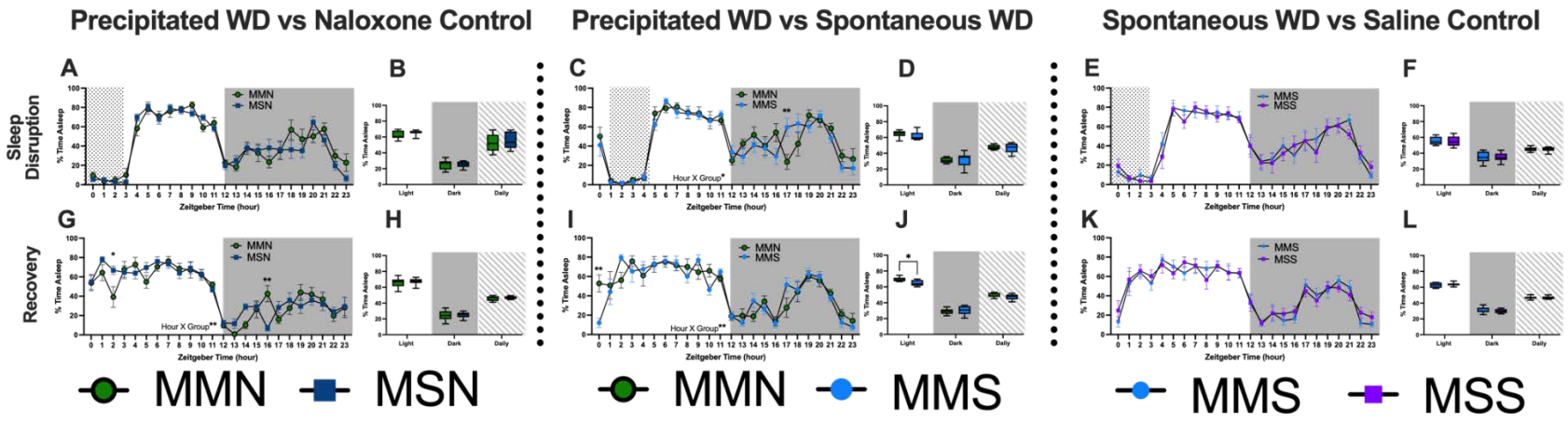
Precipitated WD vs Naloxone Control
Precipitated WD vs Spontaneous WD
Spontaneous WD vs Saline Control
Sleep Disruption
Recovery
A
B
C
D
E
F
G
H
I
J
K
L
MMN
MSN
MMS
MSS
% Time Asleep
Zeitgeber Time (hour)
Hour X Group*
Hour X Group**
Light
Dark
Daily
MMN
MSN
MMN
MMS
MMS
MSS

Figure 7. ***Sleep behavior of male mice during sleep disruption and recovery.*** Sleep percentage of male mice undergoing sleep disruption for 4 hours (**A-F**) and the day immediately following (**G-L**). Graphs are either 24-hour sleep percentages (**A, C, E, G, I, K**) or sleep percentage by light cycle (**B, D, F, H, J, L**). Columns are grouped by experiment (MMN vs MSN, MMN vs MMS, and MMS vs MSS). Grey box shows lights off and dotted region shows sleep disruption period. Each point or bar represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

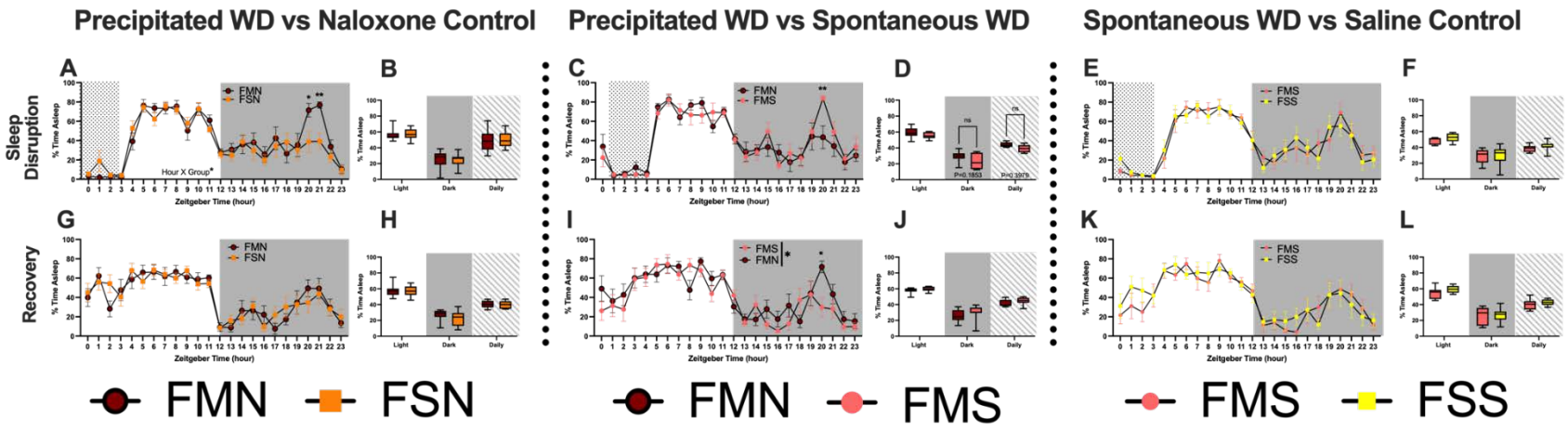
Precipitated WD vs Naloxone Control
Precipitated WD vs Spontaneous WD
Spontaneous WD vs Saline Control
Sleep Disruption
Recovery
A
B
C
D
E
F
G
H
I
J
K
L
% Time Asleep
Zeitgeber Time (hour)
Hour X Group*
FMN
FSN
FMS
FSS
Light
Dark
Daily
ns
FMN
FSN
FMN
FMS
FMS
FSS

Figure 8. ***Sleep behavior of female mice during sleep disruption and recovery.*** Sleep percentage of male mice undergoing sleep disruption for 4 hours (**A-F**) and the day immediately following (**G-L**). Graphs are either 24-hour sleep percentages (**A, C, E, G, I, K**) or sleep percentage by light cycle (**B, D, F, H, J, L**). Columns are grouped by experiment (FMN vs FSN, FMN vs FMS, and FMS vs FSS). Grey box shows lights off and dotted region shows sleep disruption period. Each point or bar represents the mean ± standard error of the mean (SEM): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

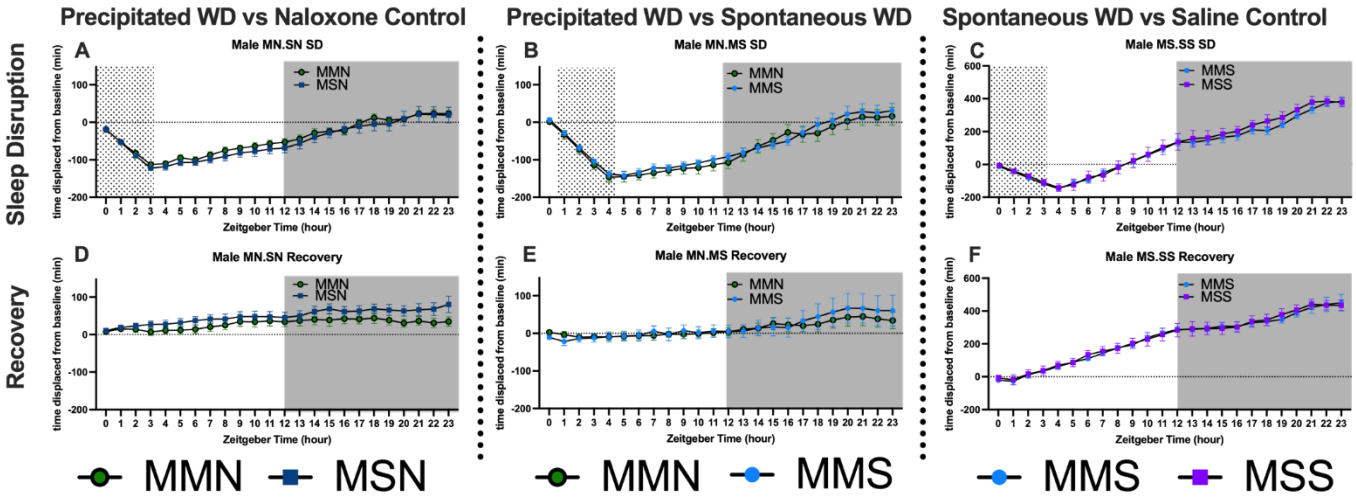

Figure 9. ***Cumulative difference in minutes of sleep of male mice during sleep disruption and recovery.*** Minutes of sleep compared to baseline sleep on sleep deprivation and recovery day. Graph shows minutes of sleep subtracted from baseline minutes, cumulative by hour. Every column is a different experiment (FMN vs FSN, FMN vs FMS, and FMS vs FSS). Grey box shows lights off and dotted region shows sleep disruption period. Graphs **C** and **F** have a different Y-axis to show all data. Each point represents the mean ± standard error of the mean (SEM).

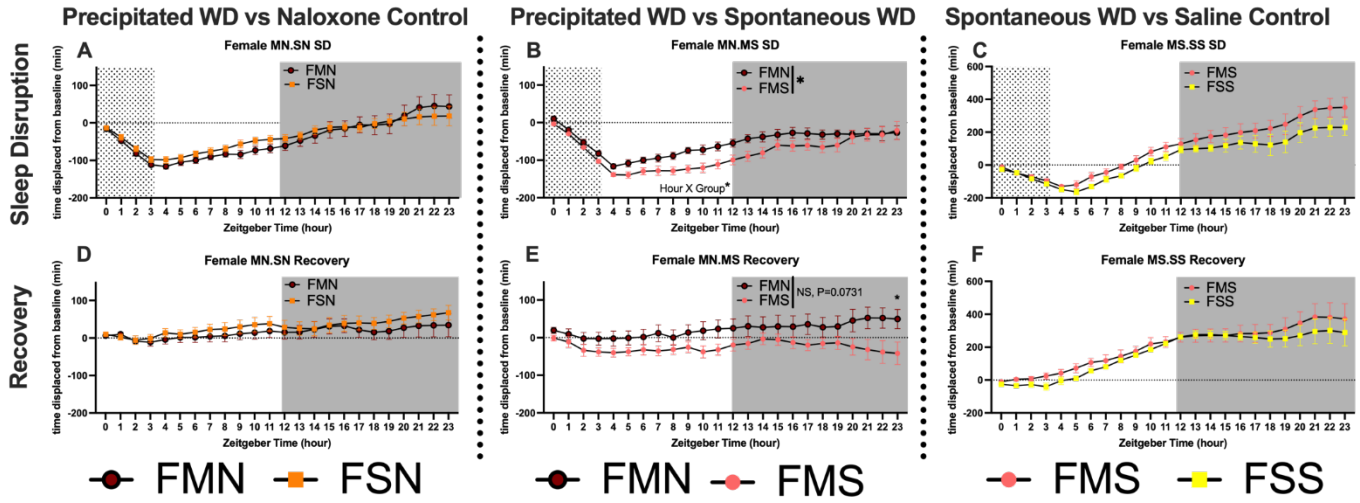

Figure 10. ***Cumulative difference in minutes of sleep of female mice during sleep disruption and recovery.*** Minutes of sleep compared to baseline sleep on sleep deprivation and recovery day. Graph shows minutes of sleep subtracted from baseline minutes, cumulative by hour. Every column is a different experiment (FMN vs FSN, FMN vs FMS, and FMS vs FSS). Grey box shows lights off and dotted region shows sleep disruption period. Graphs **C** and **F** have a different Y-axis to show all data. Each point represents the mean ± standard error of the mean (SEM): *P < 0.05.