Opioids Disrupt Sleep and Wakefulness in C57BL/6J mice

A Thesis Presented for the

Master of Arts

Degree

The University of Tennessee, Knoxville

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August 2020

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Acknowledgements

To my family and friends for the amazing love and support. My mom, Sarlenna and dad, Robert. To my brother, RJ and sisters, Kya and Haven. To my late grandparents - Clarence & Margie Dial, Earline Oxendine Locklear, and “Sweetie” Lula Jacobs. Thank you for motivating and watching over me through every step of this process.

To my esteemed laboratory colleagues, Carolina Bustamante, Zachary T. Glovak, Christopher O’Brien, Nathan Reider, Wilton Sun, & Dr. Diana Z. Unzaga, without whom this work would not have been possible. A very special thank you to my mentors, Drs. Ralph Lydic and Helen A. Baghdoyan, for their invaluable guidance and continuous support.

“The value of any undertaking is assessed in terms of the potential benefit to the human race.” – Francis Bacon

Abstract

Opioid use disorder (OUD) is a major public health burden. Opioids long have been shown to disrupt sleep and sleep disruption enhances the likelihood of addiction relapse in humans. The National Institutes of Health (NIH) refers to the mouse as one of the most powerful animal systems to study because of the genetic homologies between human and mouse. Prior to the present research, no studies have quantified the effect of opioids on states of sleep and wakefulness in mice. The current study was designed to test the hypothesis that acute administration of buprenorphine, morphine, or fentanyl disrupts sleep/wake architecture in C57BL/6J (B6) mice. Adult male mice (n = 8) were housed in a temperature-controlled environment and a 12:12 h light:dark cycle with ad libitum access to food and water. Mice were anesthetized with isoflurane for implantation of a telemeter (DSI HD-X02) and electrodes for wireless recording of cortical electroencephalogram (EEG) and dorsal neck muscle electromyogram (EMG). These signals were used to objectively quantify wakefulness, NREM sleep, and REM sleep in 10-s bins. Every mouse received at least one intraperitoneal injection (0.3 mL) each of saline (control) and an antinociceptive dose of buprenorphine, morphine, and fentanyl separated by one week. Each recording was scored independently by two individuals, one of whom was blinded to the treatment condition. Sleep scoring concordance scores were greater than 90%. These results, expressed as percent change from saline, summarize 64 recordings. Buprenorphine significantly increased wakefulness (151% ;p=0.0078) and eliminated both NREM and REM sleep. Morphine significantly increased wakefulness (142% ;p=0.0078), decreased NREM sleep (-94% ;p=0.0078) and eliminated REM sleep. Fentanyl significantly increased wakefulness (105% ;p=0.0078), decreased NREM sleep (-68% ;p=0.0078) and decreased REM sleep (-89% ;p=0.0078).

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Chapter 1: Introduction

Opioids are effective for managing pain, but clinical[1-7] and preclinical[8-11] studies show that acutely administered opioids cause sleep disruption. Chronic opioid use has been linked to a number of negative effects such as disrupted sleep architecture,[6] daytime fatigue,[5] and hypersomnolence.[1] Opioid use disorder (OUD) is a major burden on public health.[12] Medical assisted therapy for OUD includes the opioids methadone, naltrexone, and buprenorphine.[12] Characterizing the sleep disruption caused by opioids is an important step towards developing treatments for pain with reduced unwanted side effects.

Similar to breathing, sleep is a biological rhythm that is organized temporally[13] and comprised of specific neurochemical and electrophysiological changes within the brain.[14] Multiple brain regions including the brain stem, hypothalamus, and basal forebrain converge to actively produce behavioral states of wakefulness, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep.[15,16] Cortical electroencephalographic (EEG) activity associated with NREM sleep is typically within the 0.5 to 4 Hz range (i.e., delta waves) and in REM sleep it is in the 4 to 8 Hz range (i.e., theta waves).[17] Monoamines within the brain have long been known to promote wakefulness and cortical acetylcholine plays a major role in generating the active brain-states of wakefulness and REM sleep (otherwise known as paradoxical sleep because the EEG is similar to the EEG of wakefulness).[13,18] Adenosine increases in metabolically active brain regions during periods of prolonged wakefulness and suppresses wake-promoting neurotransmitters, thereby contributing to the generation of NREM and REM sleep.[13,18]

The brain's electrical[18] and neurochemical[19] activity markedly differs between states of sleep and wakefulness.[13] The physiological and temporal organization that produces healthy,

restful sleep is described as sleep architecture and provides functional metrics (i.e., latency to sleep onset, number of bouts of NREM sleep and REM sleep, and fragmentation of these episodes) for measuring sleep quality and diagnosing sleep disorders.[13,14] Sleep architecture in adult humans can be significantly altered even after a single dose of oral opioids.[2,20] Patients with chronic pain who are prescribed opioids report more sleep impairment than patients who are not prescribed opioid medications.[3] Due to the addictive and deleterious side effects of opioids, it is not ethically possible to perform mechanistic studies in humans. To overcome these limitations, non-human animals are used to fill these gaps in knowledge.

Opioids increase wakefulness and inhibit the NREM and REM phases of sleep in rats.[9] In cat, microinjection of morphine into the nucleus of the solitary tract disrupts sleep architecture.[11] Studies of rat show that morphine administered to the basal forebrain or given systemically decreases cortical acetylcholine release and slows EEG activity.[10] Buprenorphine has been shown to disrupt sleep and decrease concentrations of adenosine in sleep-regulating brain regions in rats.[8] The C57BL/6J (B6) mouse carries special relevance, as the National Institutes of Health (NIH) refers to the mouse as the premier mammalian model system for biomedical research.[21] B6 mice are genetically tractable in vivo models that facilitate mechanistic investigation into a plethora of human illnesses.[22] There is more than 90% homology between the mouse and human genome[23] supporting the view that studies of mice can help understand normal and abnormal physiology and behavior in humans. Taken together, the foregoing feasibility and translational potential described here supports the view that studies of mice offer a powerful model for investigating the neurochemical and electrophysiological characteristics regulating human sleep and wakefulness.[24]

Buprenorphine is an opioid that acts as a partial mu-opioid receptor agonist and full kappa-opioid receptor antagonist.[25] Buprenorphine has a longer half-life than fentanyl or morphine.[26] By contrast, morphine and fentanyl are full mu-opioid receptor agonists. This pharmacological difference allows buprenorphine to be a feasible and common option for substitution therapy in OUD.[25] However, sleep quality is severely impaired by stable buprenorphine or methadone treatment.[3,27] Buprenorphine also causes sleep disruption that is associated with increased depressive symptoms in clinical patients.[27]

Sleep disturbance significantly predicts the outcome of treatment for illicit drug and alcohol use disorder in primary care,[28] and many opioids cause sleep disruption that enhances the likelihood of relapse.[29] Sleep disruption can also delay wound healing[30] and increase levels of inflammatory markers such as interleukin 6.[31] Poor sleep quality has been indicated to exacerbate negative emotional states, which can lead to addiction relapse.[32] Sleep disruption can also cause hyperalgesia (increased perception of pain), which leads to higher doses of opioids required to achieve the effect of antinociception.[13,33] Taken together, these studies highlight the importance of characterizing opioid-induced sleep disruption.

A systematic review of the literature revealed no similar studies aiming to elucidate whether opioids alter states of sleep and wakefulness in the B6 mouse. This study aimed to fill this gap in knowledge by testing the hypothesis that systemic opioid administration disrupts sleep/wake states in B6 mice.

Chapter 2: Materials and Methods

Animals

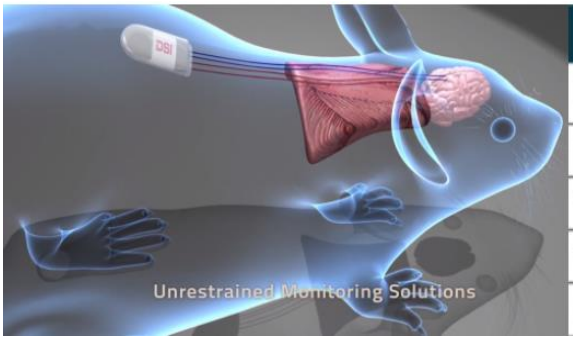
All procedures in this study were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[34] Subjects included mature, male C57BL/6J (B6) mice (Stock# 000664; n = 8) purchased from the Jackson Laboratory. Mice were housed with littermates on arrival, and cages and litter were changed weekly. Mice were given one week to adapt to the laboratory and conditioned to being handled for an additional week before beginning experiments. They were housed in a temperature-controlled environment and maintained in a 12:12 h light:dark cycle. Food and water were provided *ad libitum*. Teklad 8640 rodent chow was purchased from Research Diets, Inc., (New Brunswick, NJ, USA). At the time of the study, mice averaged 27.4 ± 1.14 (mean ± SD) weeks of age.

Telemeter implantation

The mice were anesthetized with isoflurane in order to subcutaneously implant a telemeter (HD-X02, Data Sciences International (DSI), Minneapolis, MI.) that made it possible to record electroencephalogram (EEG) and electromyogram (EMG) (Fig. 1). The telemeters were connected to bipolar electrodes implanted in the dorsal neck muscles for recording EMG. An additional pair of electrodes was implanted via craniotomies above the prefrontal cortex (A=31; L=4; V=-2) using stereotaxic coordinates of a mouse brain atlas.[35] These bipolar cortical electrodes were used to record the EEG. Mice also were implanted subcutaneously with a radio-frequency identification (RFID) chip (ID-100B 1.4, Trovan, Ltd., United Kingdom) that gave each mouse a unique identification number. During surgery, core body temperature was

monitored and maintained at 37 degrees C, and respiratory rate was recorded every 5 min. At completion of implantation, the incision was closed, and mice were placed in a warm recovery chamber where they were monitored continually until they were ambulatory. All mice recovered without difficulty and within 24 h exhibited normal feeding and grooming behaviors. After telemeter implantation the mice were housed individually throughout the remainder of the study.

The telemeters (Fig. 1), when chronically implanted wirelessly transmit recordings of EEG and EMG signals via antenna within the receiver pads beneath each mouse cage (Fig. 2). The receiver pads are connected to the DSI Matrix 2.0 (MX2) instrumentation (not pictured) via cables. This device facilitates communication between the implanted telemeter and the data acquisition computer, which was utilized to filter and analyze the transmitted signals. Once digitized, the EEG and EMG recordings were scored every 10 s for the duration of the recording as wakefulness, NREM sleep, or REM sleep.



Specifications	HD-X02
Bandwidth	0.5-80 Hz
Battery Life	1.5 months
Weight	2.2 g
Volume	1.7 cc
Minimum Subject Weight	19 g

Figure 1. Illustration of a HD-X02 telemeter implanted subcutaneously in a mouse. The drawing schematizes that electrodes placed above the cerebral cortex and in the dorsal neck muscles were connected to the telemeter (https://www.datasci.com/solutions/neuroscience?wvideo=7d1ni1696w). Manufacturer details are from Data Sciences International (DSI). Source: https://www.datasci.com/docs/default-source/implantable-telemetry/hd-x02_s02.pdf?sfvrsn=4



Figure 2. Illustration of the experimental set up for a wireless recording of sleep and wakefulness from freely moving B6 mice. Each mouse's home cage was placed on a receiver (black pad) numbered 1 to 4, which relayed the signal from the implanted telemeter to a computer in another room (not shown) where they were analyzed. This permitted the experimenter to monitor the recordings without disturbing the mice.

Recording States of Sleep and Wakefulness

After a week of surgical recovery, the signal-to-noise quality of the EEG and EMG recordings were evaluated to ensure that they could be used to objectively score states of sleep and wakefulness while mice were behaving freely in their home cage. Profiles of delta and theta power, along with EMG amplitude, made it possible to accurately score the behavioral states of sleep and wakefulness in each recording. States of wakefulness were characterized by low amplitude EEG, the presence of maximal EMG signal (Fig. 3A), and a delta/theta ratio of 1:4.[14] EEG frequencies from 0.5 to 4 Hz (delta waves) are typically seen during NREM sleep (Fig. 3B). EEG frequencies from 5 to 8 Hz (theta waves) are associated with REM sleep (Fig. 3C). These recordings created an objective index of behavioral states during each experiment.

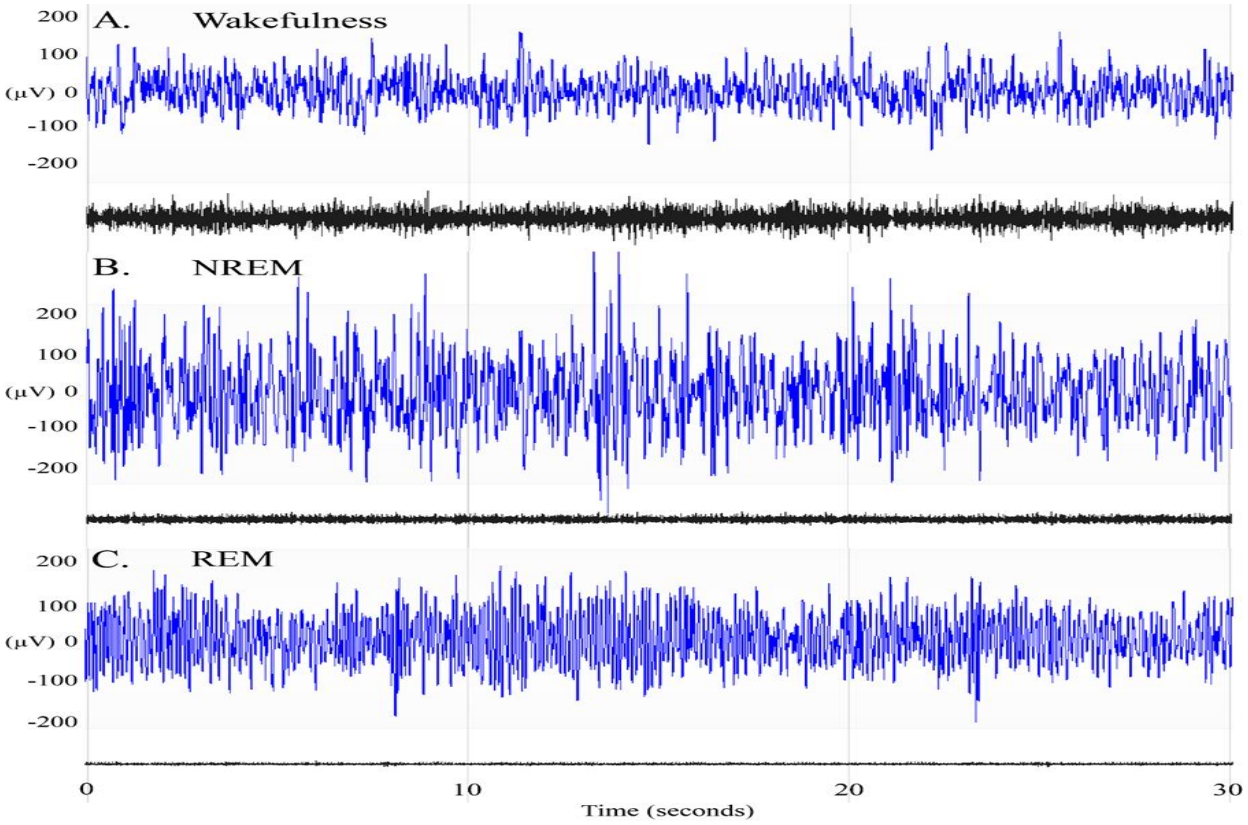


Figure 3. Illustration of EEG and EMG signals and characteristics of the behavioral states of **(A)** wakefulness, **(B)** NREM sleep, and **(C)** REM sleep. All signals were taken from the same mouse during a single recording session following injection of saline. The EEG signals are represented in blue, and the EMG signals are shown in black. The abscissa shows three sequential 10-s epochs, and the ordinate shows the amplitude of the signals (μV). Wakefulness was characterized by low amplitude/high frequency EEG combined with high amplitude EMG. NREM sleep has high amplitude/low frequency EEG (i.e., slow waves) with lower amplitude EMG, and REM sleep has low amplitude/high frequency EEG (similar to waking) and very low muscle activity shown by the EMG. Considered together, the instrumentation and recordings illustrated by Figures 1 and 2 were used to record and objectively score states of sleep and wakefulness for 4 h or 24 h after mice were injected with saline (control) or an opioid.

Experimental Design

Each mouse's body weight was recorded prior to each experiment. Mean ± SD body weight for mice used for this study was 34.8 ± 1.97 g. Data collection started immediately after the mice received intraperitoneal (IP) injections (0.3 mL) of sterile saline (control) or saline containing antinociceptive doses of buprenorphine[36] (0.3 mg/kg), morphine[37] (10 mg/kg), or fentanyl[38] (0.4 mg/kg). Recordings were performed for 4 h and began immediately post-injection. Experiments using a subset of animals (n = 4) were conducted to quantify opioid effects on sleep and wakefulness for 24 h post-injection. A within-subjects design was used such that every mouse received at least one IP injection of saline and each opioid treatment condition, which was counter-balanced regarding the order of drug administration. Mice were given one week for a washout period between injections. Data from repeated injections of the same drug into the same mouse were averaged in order not to inflate degrees of freedom. After each injection mice were returned to their home cages, which were placed on the telemeter receiving pad. Use of the DSI telemeter system made it possible to obtain recordings of sleep and wakefulness from mice freely ambulating in their home environment. Time of day for all recordings was standardized to begin 1.5 h after lights on.

Signal Processing

The EEG and EMG recordings were analyzed using the DSI Neuroscore program. The Ponemah software was set to record signals at a sampling rate of 500 Hz. EEG signals were filtered between 0.5 and 30 Hz, and EMG signals between 10 and 100 Hz. Each 4-h and 24-h recording was analyzed in 10-s epochs (i.e., 4-h recording = 1,440 epochs), which were scored for sleep and wakefulness. Epochs that contained behavioral state transitions (i.e., transitions from wakefulness to NREM sleep, NREM sleep to REM sleep, etc.) were scored based on the

behavioral state that occurred for more than half of the epoch. The Neuroscore software created a fast Fourier transformation (FFT) of the EEG signal which quantified the delta and theta EEG power for each 10-s epoch of the recording. All recordings were double-scored, and the duplicate evaluators were blinded to the treatment conditions. Recordings that did not achieve a >90% concordance between evaluators were rescored until this criterion was reached. Microsoft Excel was used to plot states of wakefulness, NREM sleep, and REM sleep as a function of time across the 4-h recording for each injection condition (Fig. 4).

Statistics Analyses

Dependent variables of this study included percent of time spent in each behavioral state, number of episodes for each state, duration of the state episodes, and latency to onset of NREM and REM sleep. These measures were quantified as the median ± interquartile range (IQR). Each of these variables represents a key component of sleep architecture and thus offered insight into which aspects of sleep were altered by the administered opioids. Descriptive and inferential statistical analyses were performed using Prism 8.3.0 (GraphPad Software, San Diego, CA). The Wilcoxon signed rank (non-parametric) test, Analysis of variance (ANOVA), and Tukey's multiple comparison test were used for post-hoc analysis. Statistical significance was shown by asterisks and defined by a probability value of (*$p<0.05$ or **$p<0.0001$).

Chapter 3: Results

3.1 Opioids altered the timing of sleep and wakefulness in B6 mice

Systemically administered opioids disrupted the sleep/wake architecture of B6 mice as illustrated by Figure 4. The temporal organization of sleep was altered for all recordings following opioid injection that were analyzed in this study. These representative plots illustrate that systemically administered opioids disrupted sleep architecture relative to saline (control). The number of transitions and episodes for each state was decreased, as shown by fewer black bars for opioid conditions when compared to saline. The duration of sleep episodes was decreased and waking episodes were increased, which is indicated by the change in the width of the black bars. The latency to onset of sleep was delayed. This is illustrated by the first sleep episodes moving further away from time point zero following administration of fentanyl, or morphine, and by the elimination of sleep caused by buprenorphine administration.

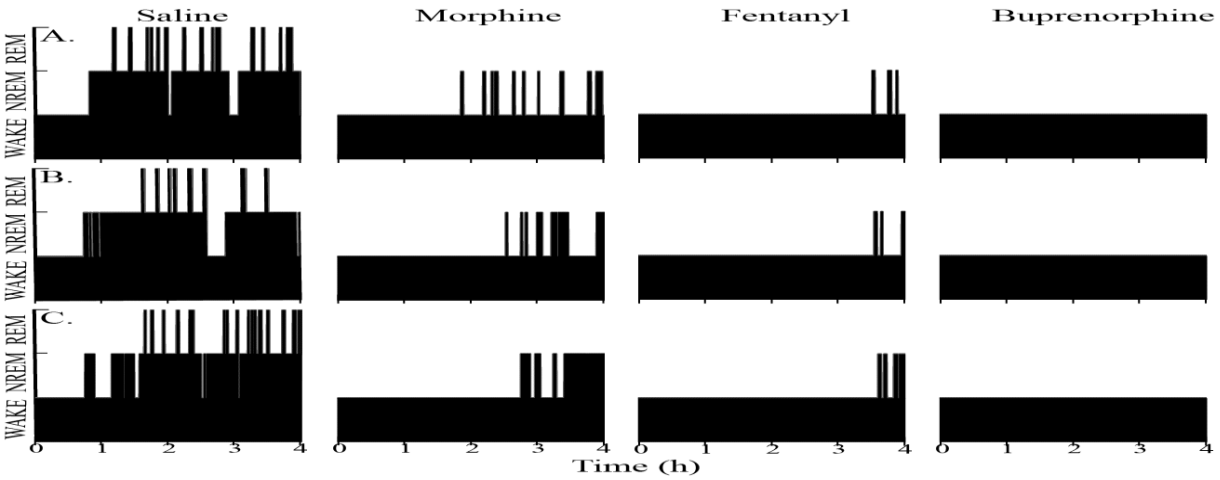


Figure 4. This figure shows the temporal organization of wakefulness (WAKE), non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep plotted as a function of time after systemic drug delivery. Each row represents the 4-h time course of sleep and wakefulness for one mouse. Each column shows injection conditions of saline (control), morphine, fentanyl,

and buprenorphine. The abscissa of each graph represents the time in hours (h) following intraperitoneal injection at time point zero. The ordinate indicates the behavioral states of wakefulness, NREM sleep, and REM sleep. The histogram height provides information about whether the mouse was awake or asleep, and the width of each histogram indicates the duration of time spent in each state.

3.2 Opioids altered the percent of 4-h recoding time in states of sleep and wakefulness

The percent of time spent in each measured behavioral state as a function of the drug condition is summarized for 4-h recordings in Figure 5.

3.3 Opioids caused disruption of sleep and wakefulness lasting up to 16 hours

A subset of animals (n = 4) was used to quantify percent of time spent in states of wakefulness, NREM sleep, and REM sleep for 24 h post-injection. These studies aimed to determine the duration of the opioid effects on sleep and wakefulness. Sleep/wake cycles in mammals are closely tied to cycles of light and dark (i.e., humans are diurnal and mice are nocturnal), so it was important to quantify the recordings during periods of light (7:00 a.m. to 7:00 p.m.) and dark (7:00 p.m. to 7:00 a.m.). The change in the percent of time spent in each measured behavioral state as a function of the drug condition is summarized for 24-h post-injection in Figure 6.

3.4 Opioids disrupted the temporal organization of 24-h sleep/wake architecture

The time course (in 2-h intervals) for percent of time spent in each measured behavioral state as a function of the drug condition is summarized for 24-h post-injection in Figure 7.

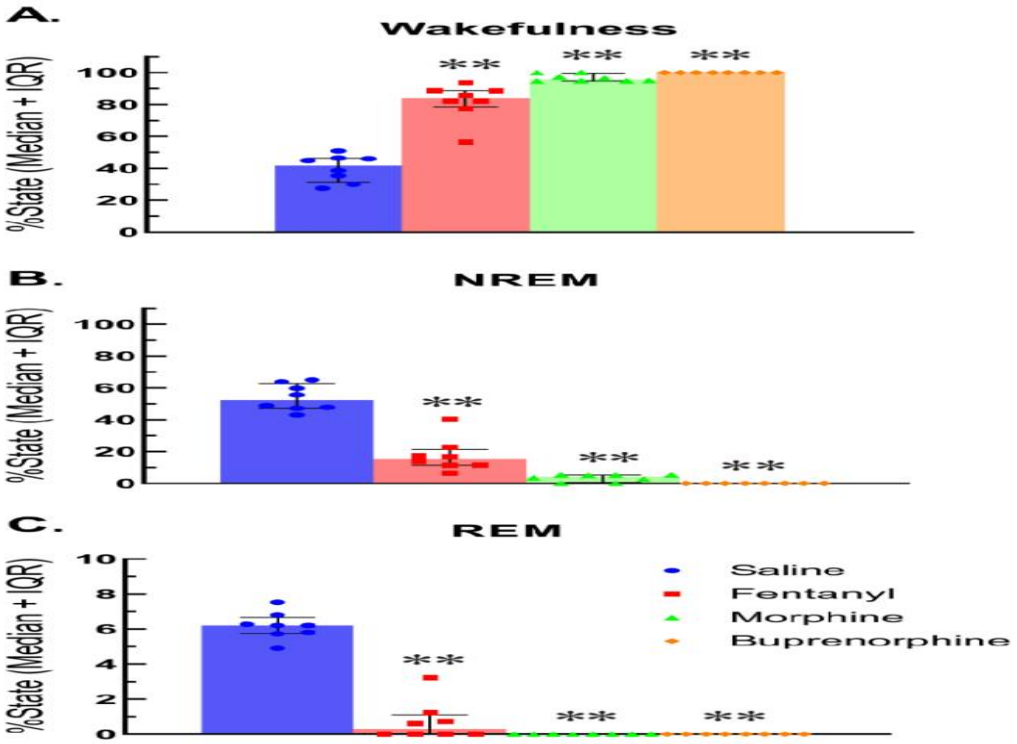


Figure 5. This figure illustrates the group data for percent of time spent in behavioral states of sleep and wakefulness as a function of the treatment condition. The data points above each histogram represent data from individual mice (n = 8). Histogram height conveys the median. Black lines between the data points illustrate the interquartile range (IQR). **(A)** One-way ANOVA showed that mice spent significantly more time in wakefulness after injection of each opioid when compared to saline ($F=118.2$; $df=3,28$; $p<0.0001$). **(B)** indicates NREM sleep was significantly decreased by morphine and fentanyl and was eliminated by buprenorphine ($F=106.5$; $df=3,28$; $p<0.0001$). **(C)** illustrates that mice spent significantly less time in REM sleep, which was eliminated by both morphine and buprenorphine ($F=155.4$; $df=3,28$; $p<0.0001$). Across 4 h, opioids caused an increase in wakefulness and a decrease in NREM and REM sleep compared with saline. Tukey's multiple comparison test revealed significant (**$p<0.0001$) differences for all three opioid treatment conditions relative to saline during a 4-h recording.

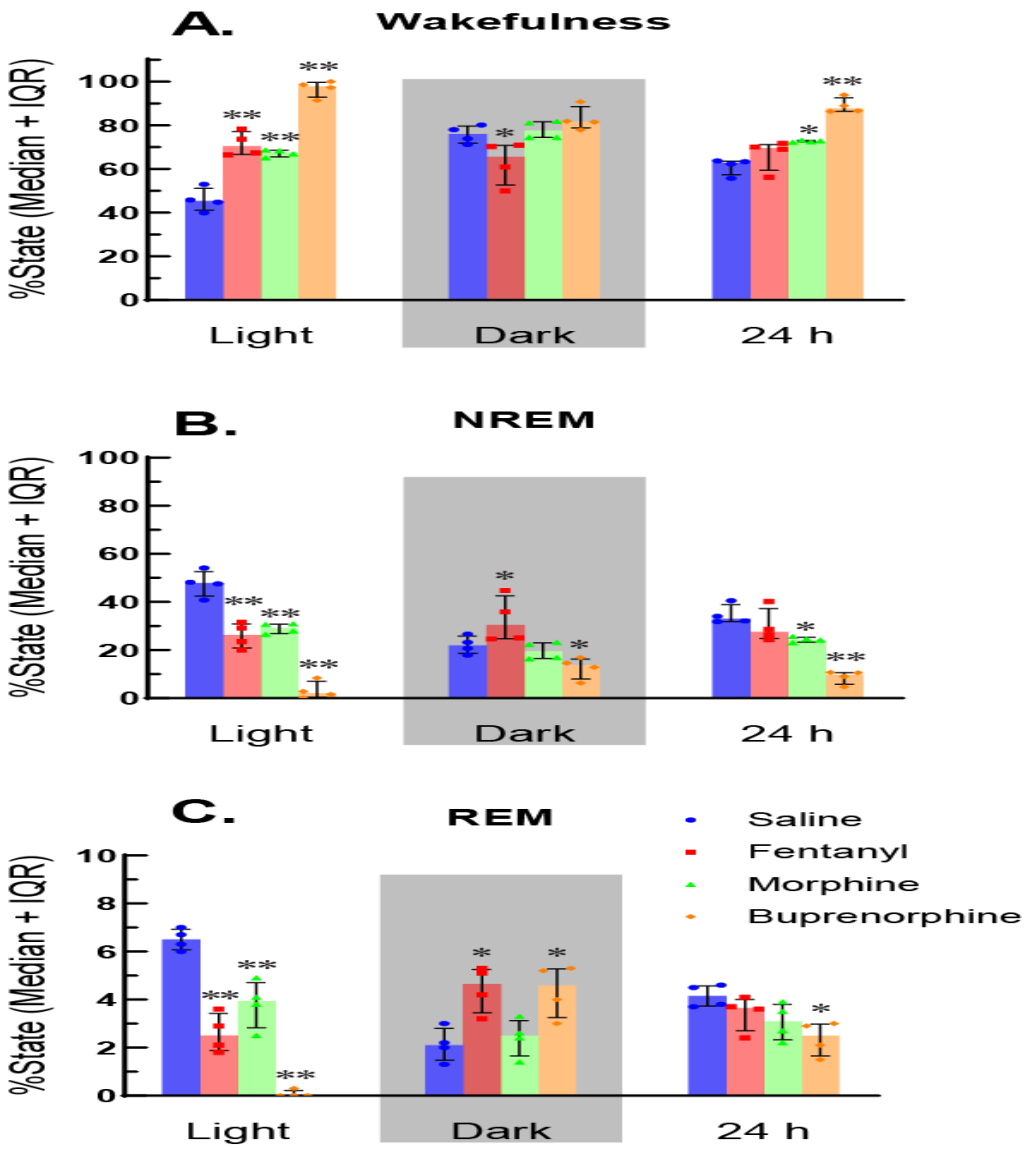


Figure 6. This figure illustrates that opioids significantly altered the percent of time spent in each behavioral state during the light phase, the dark phase, and across the 24-h period. Two-way ANOVA revealed significant main effects and an interaction due to injection condition and light/dark phases for percent of 24-h recording spent in behavioral states. On the ordinate of every graph is the percent of time spent in each behavioral state plotted as a function of injection condition. Histogram height represents the median and the black lines convey the IQR. The abscissa of each graph shows the 24-h period and both the light (7:00 a.m. to 7:00 p.m.) and dark

(gray shading indicating 7:00 p.m. to 7:00 a.m.) periods. **(A)** Two-way ANOVA revealed mice spent significantly more time in wakefulness following opioid injection relative to saline ($F=42.17$; $df=3,24$; $p<0.0001$); light vs dark comparison revealed a significant main effect ($F=5.745$; $df=1,24$; $p=0.0247$), and evaluation of the interaction indicated significant differences as a function of drug and light/dark phase ($F=26.83$; $df=3,24$; $p<0.0001$). **(B)** indicates that NREM sleep was significantly decreased by opioids compared to saline ($F=40.02$; $df=3,24$; $p<0.0001$). There was a significant effect of light/dark phase ($F=6.533$; $df=1,24$; $p=0.0173$) and a significant interaction between injection condition and light/dark phase ($F=19.37$; $df=3,24$; $p<0.0001$). **(C)** illustrates that mice exhibited significantly less REM sleep following opioid injection when compared with saline during the 24-h period and the light/dark cycles ($F=9.482$; $df=3,24$; $p=0.0003$). Light vs dark comparisons revealed no differences, and evaluation of the interaction between injection condition and light/dark phases indicated significant differences ($F=45.04$; $df=3,24$; $p<0.0001$). Across 24 h, opioids caused an increase in wakefulness and a decrease in NREM and REM sleep compared with saline. Tukey's multiple comparison test revealed significant (*$p<0.05$ and **$p<0.0001$) differences for percent state following morphine and buprenorphine relative to saline for the 24-h period, though there were no differences following acute fentanyl administration. There were significant (**$p<0.0001$) differences for percent of time in behavioral states following all three opioid treatment conditions compared with saline during the light cycle. Percent of time spent in each behavioral state following fentanyl or buprenorphine administration was significantly (*$p<0.05$) different from saline during the dark cycle, however there were no morphine effects on sleep and wakefulness during the dark cycle.

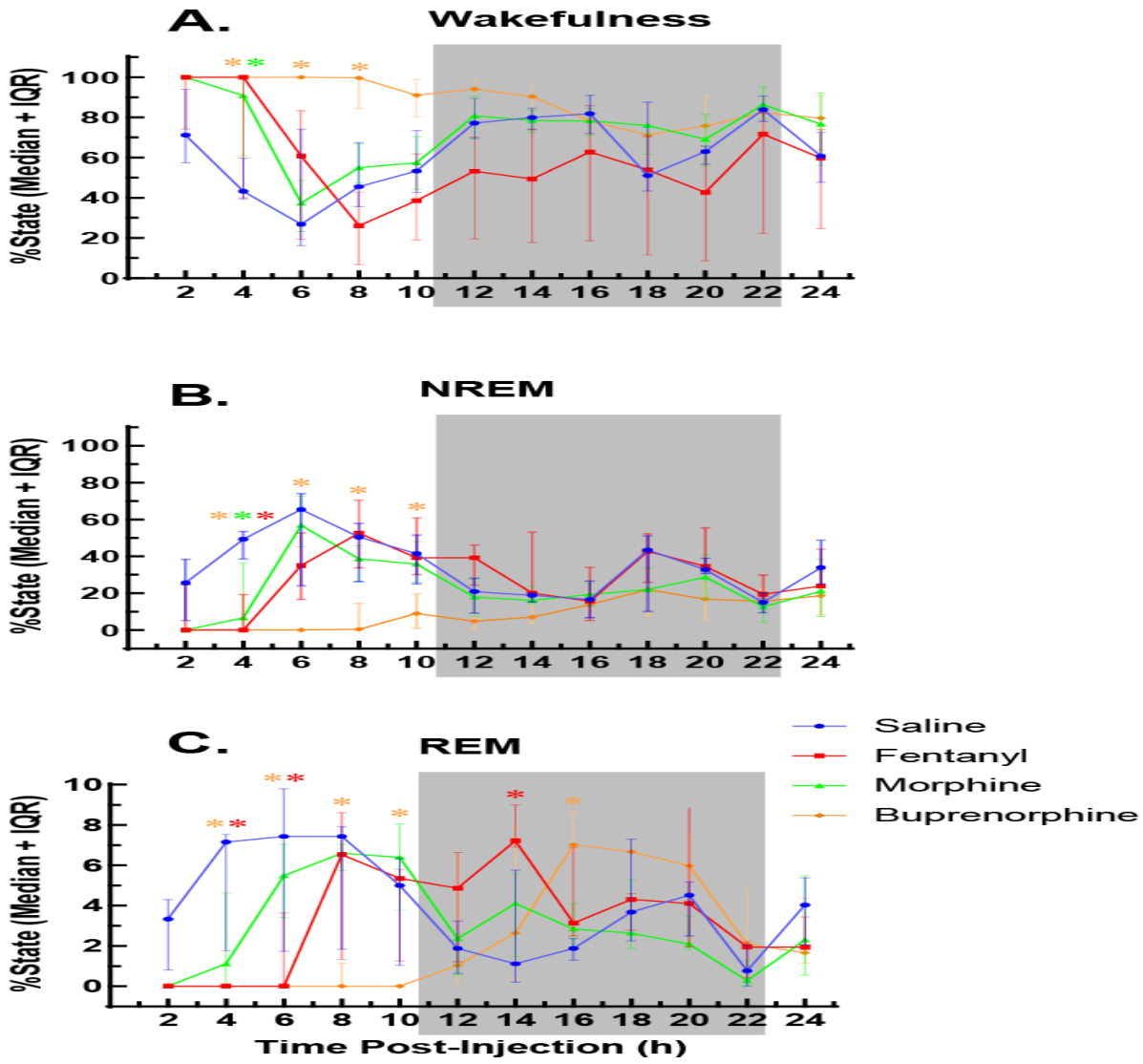


Figure 7. Illustration of the time course for percent sleep and wakefulness for (n = 4) mice during 24-h recordings. This aids in characterizing behavioral states as altered by each of the four injection conditions. The differences in time course for percent state of wakefulness, NREM sleep, and REM sleep across a 24-h recording are plotted in 2-h time intervals for each drug condition. This figure illustrates 10.5 h of light, followed by 12 h of dark, and 1.5 h of light that comprised the 24-h recording. The data points on the time-course plot represent the median and the IQR. The shaded area from 10.5 h to 22.5 h post-injection indicates the dark period. Two-way ANOVA evaluated the potential for main effects on state due to drug, time post-injection,

and for a drug by time interaction **(A)**. Mice spent significantly more time in wakefulness after opioid injection as a function of time **(**$F=4.77$; $df=11,144$; $p<0.0001$) and drug **(**$F=28.21$; $df=3,144$; $p<0.0001$). There was also a significant drug by time interaction for the percent of time in wakefulness **(**$F=1.842$; $df=33,144$; $p=0.0077$). **(B)** ANOVA revealed mice spent significantly less time in NREM sleep as a function of time **(**$F=7.495$; $df=11,144$; $p<0.0001$) and drug **(**$F=27.92$; $df=3,144$; $p<0.0001$). NREM sleep was significantly decreased as a function of the interaction between injection condition and time **(**$F=2.578$; $df=33,144$; $p<0.0001$). **(C)** illustrates that mice spent significantly less time in REM sleep following opioid injection as a function of drug **(**$F=1.897$; $df=3,144$; $p<0.0001$). ANOVA revealed there was no time effect on REM sleep, but the drug by time interaction was significant ($F=3.556$; $df=33,144$; $p<0.0001$). Tukey's multiple comparison test revealed significant ($*p<0.05$) differences in the time course of behavioral states following acute opioid administration when compared with saline at each 2-h time segment. Asterisks, color-coded utilizing the figure legend for fentanyl, morphine, and buprenorphine, were placed above each 2-h time interval. This illustrated opioid treatment conditions that caused a significant ($*p<0.05$) change in sleep and wakefulness, relative to saline, at each 2-h period. For instance, the orange asterisks at 4-h post-injection for every measured behavioral state shows that buprenorphine significantly ($*p<0.05$) increased wakefulness (relative to saline in blue) and deceased both NREM and REM sleep during this 2-h interval. NREM sleep following fentanyl or morphine was significantly ($*p<0.05$) different from saline levels at 4 h and 6 h post-injection, respectively. After buprenorphine injection, REM sleep required approximately 10 h to return to saline levels. Post-administration of fentanyl or buprenorphine mice spent significantly ($*p<0.05$) more time in REM sleep (relative to saline) at 14 h and 16 h post-injection, respectively, during the dark cycle.

3.5 Opioids altered the amount of time spent in states of sleep and wakefulness during periods of light and dark

The percent change for time mice spent in each measured behavioral state as a function of the injection condition is summarized for the light and dark cycles in Figure 8.

3.6 Opioids decreased the number of episodes of wakefulness, NREM sleep, and REM sleep

The difference in the number of episodes for each measured behavioral state as a function of the drug condition 4-h post-injection is summarized in Figure 9.

3.7 Opioids decreased the number of episodes of sleep and wakefulness during a 24-h recording

Differences in the number of episodes for each measured behavioral state as a function of the drug condition 24-h post-injection are summarized in Figure 10.

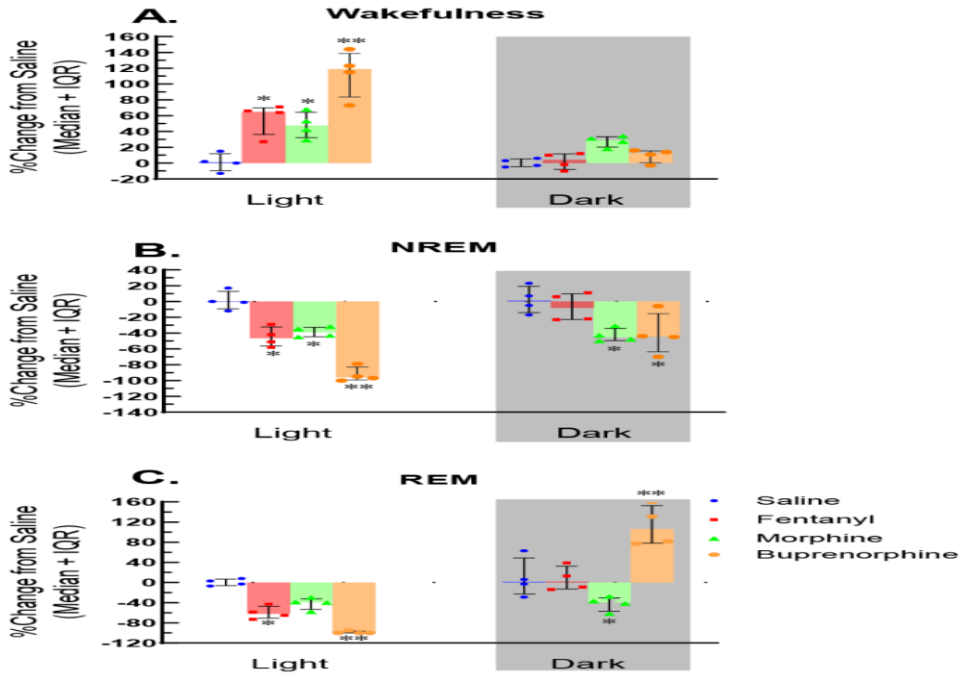


Figure 8. Illustration of the percent change (from saline) for each measured behavioral state

during the light and dark periods following opioid administration. The points above each histogram represent data from each mouse (n = 4). On the ordinate of every graph is the percent change relative to saline for amount of time spent in each behavioral state as a function of the injection condition. The top of each histogram represents the median and the black lines convey the IQR. The abscissa of each graph shows both the light (7:00 a.m. to 7:00 p.m.) and dark (7:00 p.m. to 7:00 a.m.) phases. **(A)** One-way ANOVA showed that mice spent significantly more time in wakefulness as a function of drug ($F=20.7$; $df=3,24$; $p<0.0001$) and revealed a significant main effect of light/dark cycle **(**$F=66.21$; $df=1,24$; $p<0.0001$). The drug by time interaction was also significant regarding the time spent in wakefulness **(**$F=16.78$; $df=3,24$; $p<0.0001$). **(B)** indicates that NREM sleep was significantly decreased by opioids compared to saline ($F=28.91$; $df=3,24$; $p<0.0001$), and there was a significant main effect of light/dark period ($F=16.41$; $df=1,24$; $p=0.0005$). There was also a significant drug by time interaction for the percent of time in NREM sleep **(**$F=6.634$; $df=3,24$; $p=0.002$). **(C)** illustrates that mice spent significantly less time in REM sleep following opioid injection when compared to saline ($F=8.672$; $df=3,24$; $p=0.0004$). There was a significant main effect of light/dark cycle **(**$F=77.67$; $df=1,24$; $p<0.0001$) and drug by time interaction **(**$F=36.01$; $df=3,24$; $p<0.0001$) regarding the time in REM sleep. Tukey's multiple comparison test revealed significant (*$p<0.05$ and **$p<0.0001$) differences for all three opioid conditions compared to saline for the light cycle. Following morphine and buprenorphine administration, NREM and REM were significantly (*$p<0.05$ and **$p<0.0001$) different from saline during the dark cycle. Fentanyl had no effect on any of the three measured behavioral states during the dark phase.

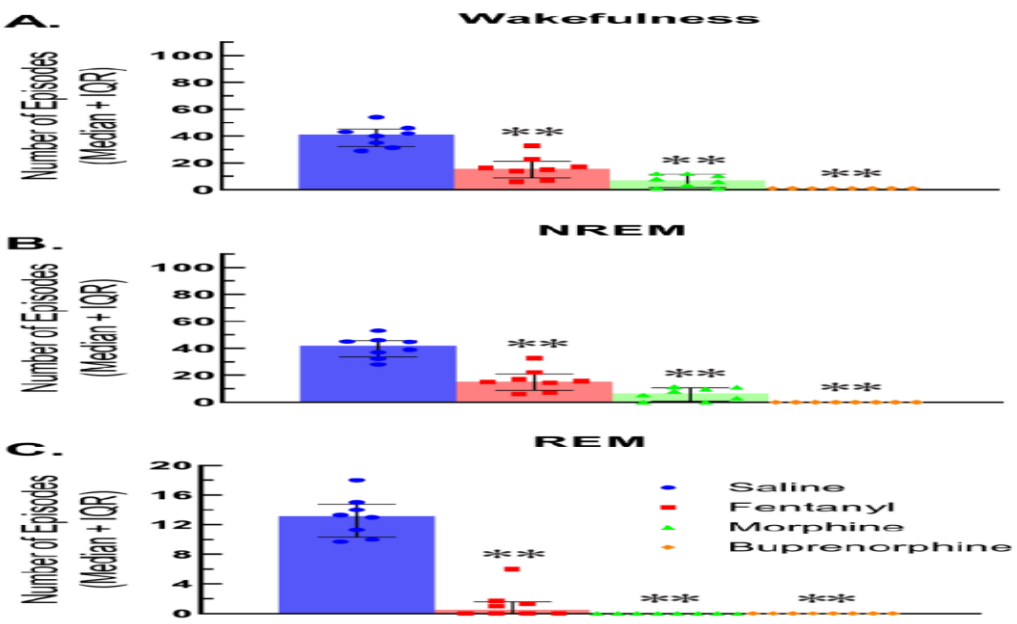


Figure 9. This figure shows the decrease in the total number of waking, NREM, and REM sleep episodes following opioid administration when compared to saline during the 4-h recordings for (n = 8) mice . The total number of episodes for each behavioral state was decreased by opioids. The ordinate of each graph illustrates the number of episodes as a function of drug condition. Histogram height represents the median and the black lines convey the IQR. **(A)** shows that opioids caused a significant decrease in the number of waking episodes relative to saline ($F=59.06$; $df=3,28$; $p<0.0001$). **(B)** The number of NREM sleep episodes was significantly decreased by morphine and fentanyl and was eliminated by buprenorphine ($F=64.38$; $df=3,28$; $p<0.0001$). **(C)** The number of REM episodes was significantly decreased by all opioids ($F=109.8$; $df=3,28$; $p<0.0001$). REM sleep episodes were eliminated by both morphine and buprenorphine. Across 4 h, following opioid injection mice showed a decrease in the number of waking, NREM sleep, and REM sleep episodes compared with saline. Tukey's multiple comparison test revealed significant (**$p<0.0001$) differences, relative to saline, in the number of episodes for every measured behavioral state following fentanyl, morphine, or buprenorphine.

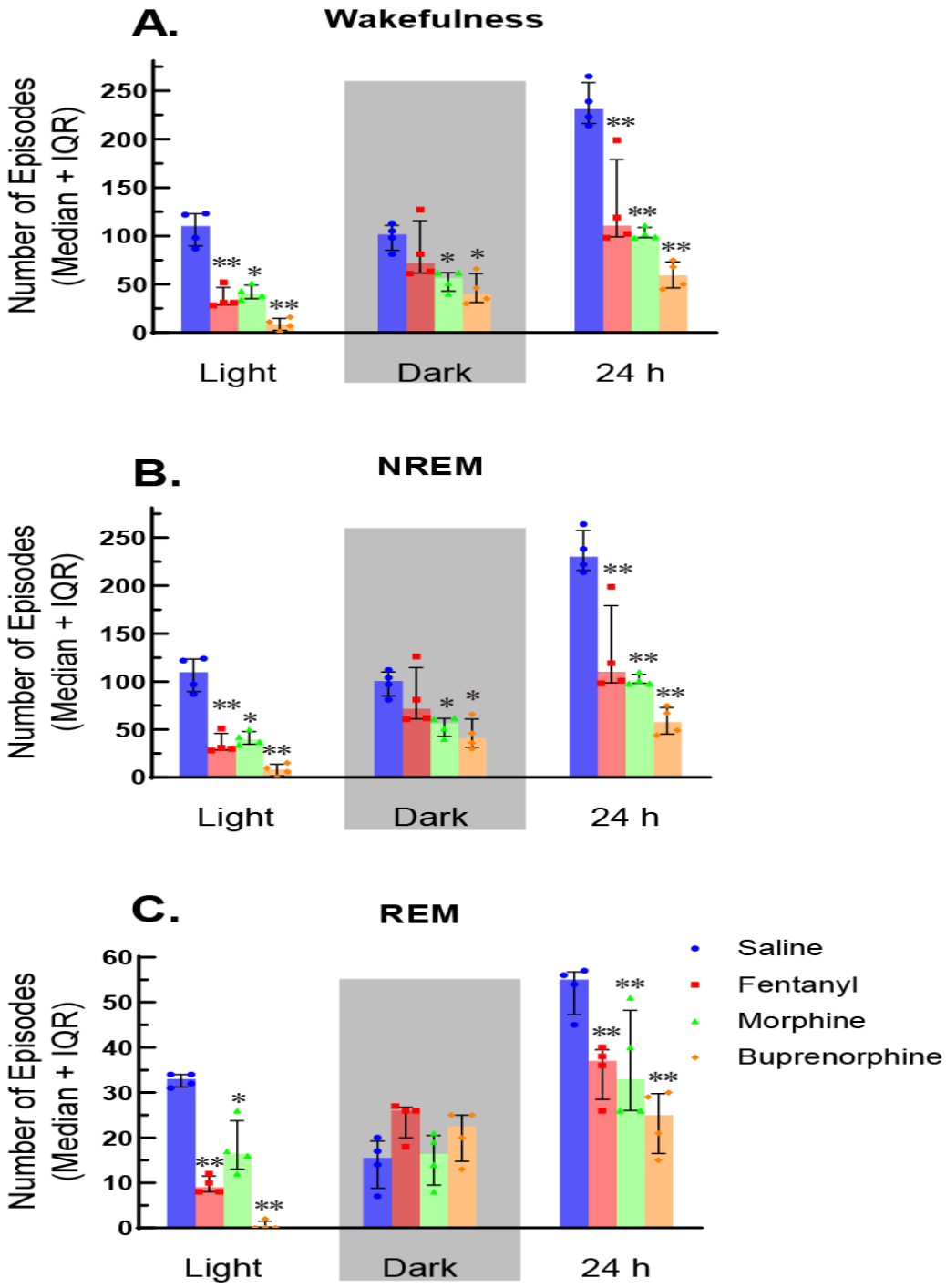


Figure 10. This figure illustrates opioids significantly reduced the number of episodes for each behavioral state during a 24-h period, and the light and dark phases. Two-way ANOVA revealed significant main effects and an interaction due to injection condition and light/dark phases for the number of episodes of wakefulness, NREM, and REM sleep during a 24-h recording. The data

points above each histogram represent each mouse (n = 4). On the ordinate of every graph is the number of episodes for each behavioral state as a function of injection condition. Histogram height represents the median, and the IQR is shown by the black bars. The abscissa of each graph shows the 24-h period and both the light (7:00 a.m. to 7:00 p.m.) and dark (7:00 p.m. to 7:00 a.m.) cycles, which is also represented by the shaded box. **(A)** shows mice exhibited significantly fewer waking episodes after each opioid injection when compared to saline ($F=33.15$; $df=3,24$; $p<0.0001$). There was a significant main effect of light/dark phase ($F=14.91$; $df=1,24$; $p=0.0007$) and interaction between injection condition and light/dark phase for the number of waking episodes ($F=4.84$; $df=3,24$; $p=0.009$). **(B)** indicates the number of NREM sleep episodes was significantly decreased by opioids during a 24-h recording ($F=33.75$; $df=3,24$; $p<0.0001$). Light vs dark comparisons revealed a significant main effect ($F=15.52$; $df=1,24$; $p=0.0006$), and evaluation of the interaction was also significant regarding the number of NREM sleep episodes ($F=5.115$; $df=3,24$; $p=0.0071$). **(C)** illustrates that mice had significantly fewer REM sleep episodes after each opioid injection when compared to saline ($F=11.55$; $df=3,24$; $p<0.0001$). ANOVA revealed a significant main effect of light/dark phase ($F=5.38$; $df=1,24$; $p=0.0292$), and interaction between injection condition and light/dark phase for the number of REM sleep episodes ($F=31.15$; $df=3,24$; $p<0.0001$). Across 24 h, following opioid injection mice showed a decrease in the number of waking, NREM sleep, and REM sleep episodes when compared with saline. Tukey's multiple comparison test revealed significant ($*p<0.05$ and $**p<0.0001$) differences for all three opioid treatment conditions compared with saline during the light cycle and 24-h period. Behavioral states following administration of morphine or buprenorphine were significantly ($*p<0.05$) different from saline during the dark cycle. There was no effect of fentanyl on the number of episodes of sleep or wakefulness during the dark period.

3.8 Opioids altered the duration of time mice spent in episodes of sleep and wakefulness

The change in the duration of the episodes for the measured behavioral states of wakefulness, NREM, and REM sleep as a function of the drug condition is summarized for 4-h recordings in Figure 11.

3.9 Opioids disrupted the duration of episodes of sleep and wakefulness during a 24-h recording

Changes in the duration of the episodes for each behavioral state as a function of the drug condition are shown for the 24-h period post-injection in Figure 12.

3.10 Opioids delayed the onset of NREM sleep and REM sleep

The change in the latency to onset of sleep as a function of the drug condition is summarized for the 4-h period post-injection in Figure 13. 24-h recordings shown in Figure 14 made it possible to quantify the sleep latency that was eliminated by morphine (i.e., REM sleep) and buprenorphine (i.e., NREM and REM sleep).

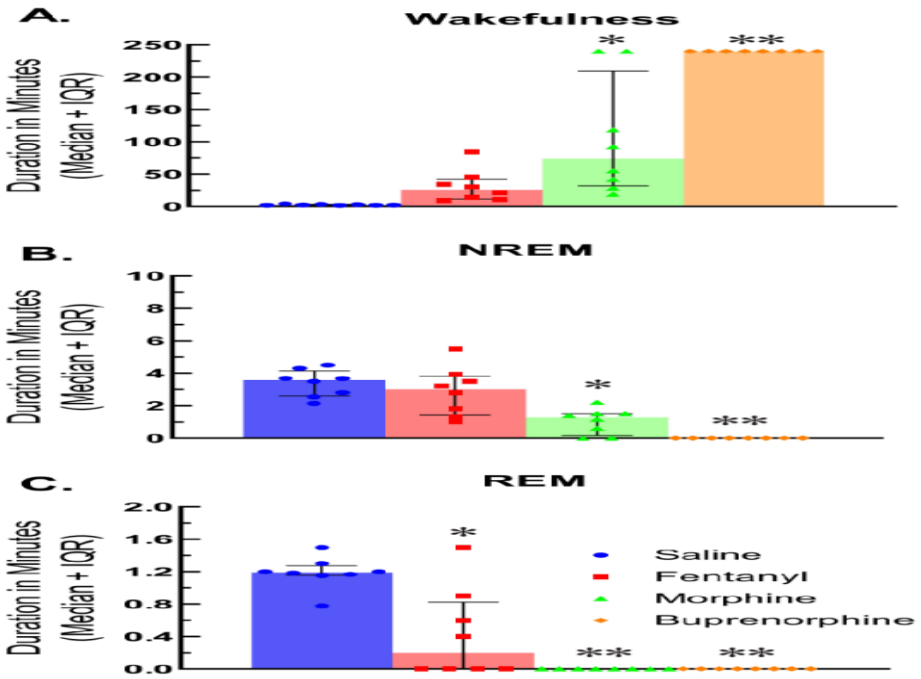


Figure 11. This figure summarizes the difference in duration (in minutes) of states of wakefulness, NREM, and REM sleep following saline or opioid administration. The duration of the episodes for each behavioral state during a 4-h recording was disrupted by systemic opioids. The data points represent each mouse (n = 8). The ordinate of every graph illustrates the duration of the episodes (in minutes) as a function of drug represented as the median (histogram height) and IQR (black lines). **(A)** shows that compared to saline, opioids caused significantly longer waking episodes ($F=41.35$; $df=3,28$; $p<0.0001$). **(B)** indicates the duration of NREM sleep episodes was significantly decreased by morphine and these episodes were eliminated by buprenorphine ($F=22.68$; $df=3,28$; $p<0.0001$). **(C)** the duration of REM sleep episodes was significantly decreased by fentanyl and eliminated by both morphine and buprenorphine ($F=28.98$; $df=3,28$; $p<0.0001$). Across 4 h, following opioid injection mice showed an increase in the duration of waking episodes and a decrease in the duration of NREM and REM sleep episodes when compared with saline. Tukey's multiple comparison test revealed significant (*$p<0.05$ and **$p<0.0001$) differences for the duration of episodes of each behavioral state between saline and all opioid treatment conditions. There was no effect of fentanyl on the duration of waking and NREM sleep episodes during a 4-h recording.

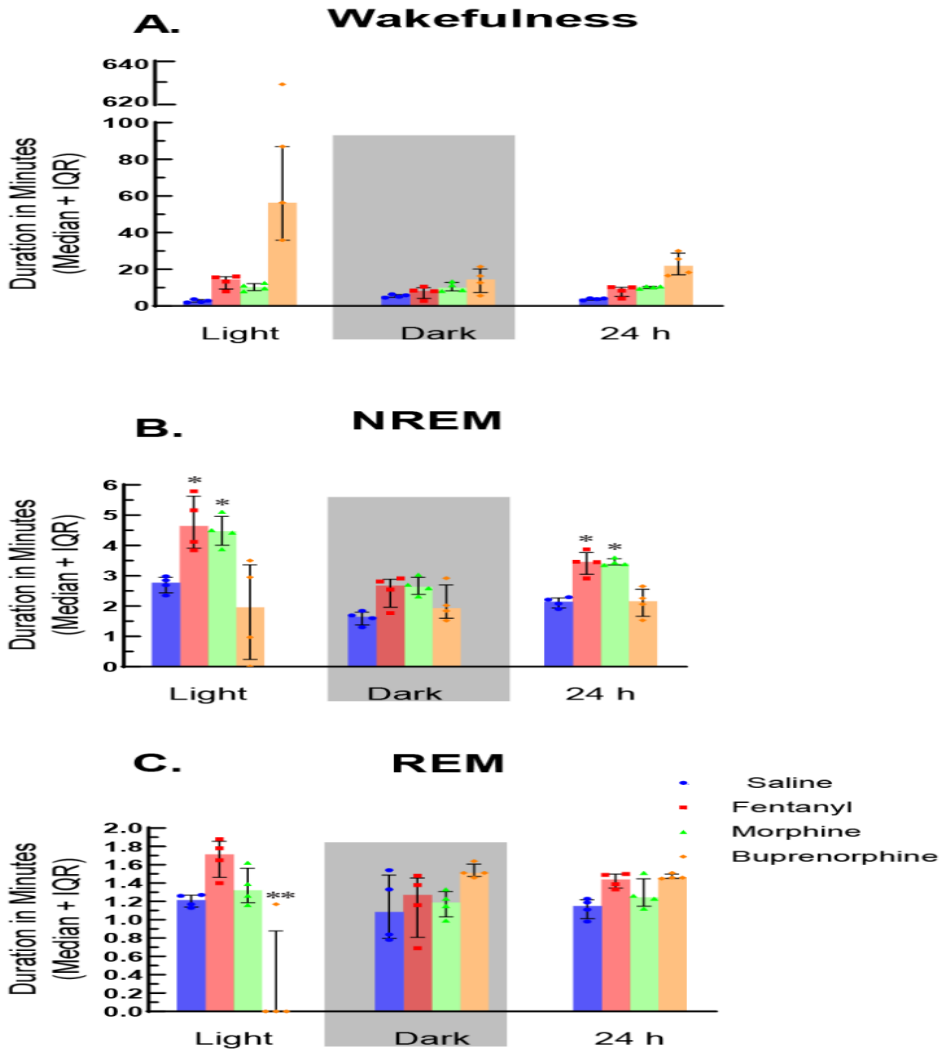


Figure 12. Illustration of the change in duration (in minutes) for each measured behavioral state as a function of the drug condition during the light, dark, and 24-h phases. Two-way ANOVA revealed potential main effects and an interaction due to injection condition and light/dark phases on the duration of episodes during a 24-h period. The data points above each histogram represent each mouse (n = 4). On the ordinate of every graph is the duration of episodes in minutes for each behavioral state as a function of the injection condition. Histogram height represents the median, and the IQR is shown by the black bars. The abscissa of each graph shows the 24-h period and both the light (7:00 a.m. to 7:00 pm) and dark (7:00 pm to 7:00 a.m.) cycles,

which is also represented by the shaded box. **(A)** shows that the trend across 24 h is for acute opioid administration to increase the duration of waking episodes relative to saline. The median (and IQR) for the duration of waking episodes following buprenorphine administration were calculated (and illustrated above) excluding the outlier data point at 630 minutes during the light cycle. Two-way ANOVA included data from every mouse and revealed no effect on the duration of waking episodes as a function of drug, time, or the drug by time interaction during a 24-h recording. **(B)** indicates the duration of NREM sleep episodes was significantly disrupted by opioids ($F=10.88$; $df=3,24$; $p=0.0001$), and there was a significant main effect of light vs dark cycle ($F=20.95$; $df=1,24$; $p=0.0001$). Evaluation of the interaction between injection condition and light/dark phases was also significant for the duration of NREM episodes ($F=3.959$; $df=3,24$; $p=0.02$). **(C)** illustrates the duration of REM sleep episodes was significantly disrupted by opioids ($F=4.177$; $df=3,24$; $p=0.0163$). There was no effect of light/dark phase on the duration of REM sleep episodes. The interaction between injection condition and light/dark phases was also significant regarding the duration of REM episodes ($F=13.13$; $df=3,24$; $p<0.0001$). Across 24 h, following opioid injection mice showed an increase in the duration of waking episodes and decrease in the duration of NREM and REM sleep episodes when compared with saline. Tukey's multiple comparison test revealed significant ($*p<0.05$ and $**p<0.0001$) differences for all three opioid treatment conditions relative to saline during the light cycle and 24-h period. However, there was no effect on the duration of episodes for each behavioral state during the dark cycle following injection of fentanyl, morphine, or buprenorphine when compared with saline.

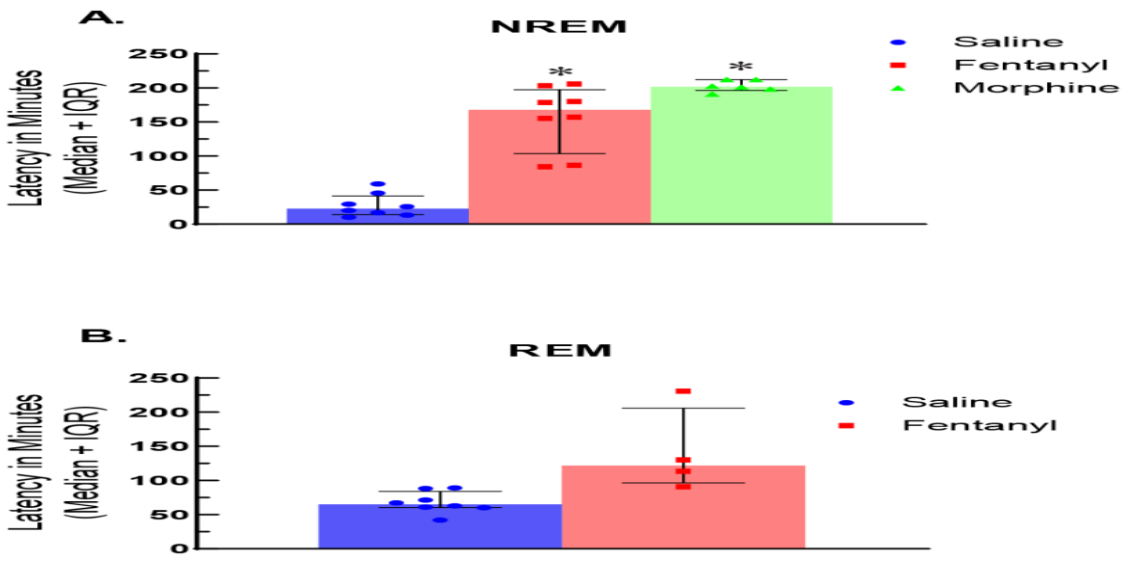


Figure 13. This figure shows the increase in latency to onset of NREM and REM sleep caused by acute administration of saline, fentanyl, or morphine. Wilcoxon signed rank test revealed opioids significantly increased the time from drug administration to the onset of sleep. Recordings which did not contain NREM (i.e., n = 8 post-buprenorphine and n = 2 following morphine) or REM sleep (i.e., n = 8 post-buprenorphine or morphine, and n = 4 following fentanyl) were excluded from the Fig. 13 summary. On the ordinate of every graph is the latency to onset of sleep (in minutes) as a function of drug represented as the median and IQR. **(A)** Wilcoxon signed rank test showed the latency to onset of NREM sleep was significantly increased following injection of fentanyl (Z=2.42; Mdn=141.1; p=0.0078) or morphine (Z=1.86; Mdn=183.7; p=0.0313) relative to saline. NREM sleep was eliminated by buprenorphine and during n = 2 morphine (4-h) recordings. **(B)** shows REM sleep was eliminated following injection of fentanyl in n = 4 recordings. Wilcoxon signed rank test revealed there was no change in the latency to onset of REM sleep in the remaining (n = 4) recordings of sleep and wakefulness post-administration of fentanyl. Morphine and buprenorphine eliminated REM sleep in 4-h recordings of sleep of wakefulness. Across 4 h, opioid administration caused an increase in the latency to onset of NREM and REM sleep.

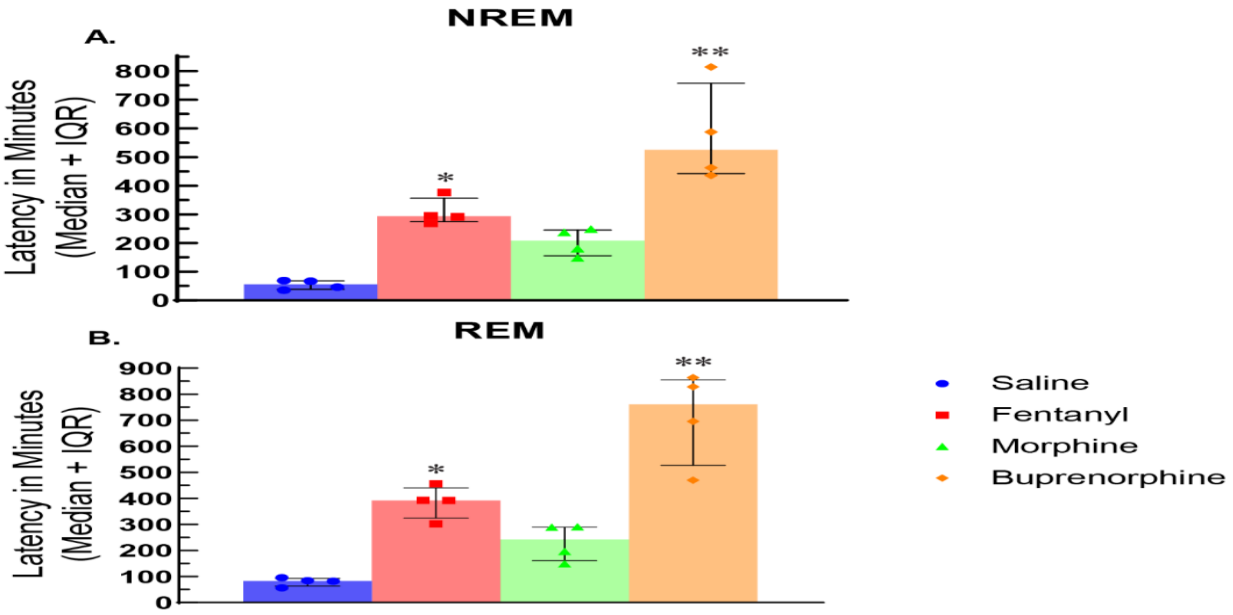


Figure 14. Illustration of the increase in latency to onset of NREM and REM sleep as a function of the drug condition for the 24-h recordings in (n = 4) mice. One-way ANOVA showed the latency to onset of NREM and REM sleep was delayed by systemic opioids. On the ordinate of every graph is the latency to onset of sleep (in minutes) as a function of drug. Histogram height represents the median and the black bars show the IQR. **(A)** shows a significant increase in the latency to NREM sleep onset following injection of opioids when compared with saline ($F=22.3$; $df=3,12$; $p<0.0001$). **(B)** shows latency to onset of REM sleep was significantly increased by fentanyl, morphine, or buprenorphine ($F=28.78$; $df=3,12$; $p<0.0001$). Across 24 h, following opioid injection mice showed an increase in latency to onset of NREM and REM sleep relative to saline. Tukey's multiple comparison test revealed significant (*$p<0.05$ and **$p<0.0001$) differences in the latency to onset of sleep following administration of fentanyl and buprenorphine, respectively. Morphine had no effect on the onset to latency of NREM and REM sleep during a 24-h recording.

Chapter 4: Discussion

Opioids are effective for pain management. However, all opioids cause many unwanted side effects including respiratory depression,[39,40] delayed wound healing,[30] and disruption of sleep.[1,2,6] The purpose of this study was to determine the effects of systemically administered morphine, fentanyl, and buprenorphine on states of sleep and wakefulness in B6 mice. The main finding of this study is that all measures of sleep in B6 mice were disrupted by acute opioid administration. Furthermore, the largest disruption in sleep was caused by buprenorphine, which is FDA approved for treatment for OUD.[12] Previous literature has indicated that sleep disruption is the number one predictor of relapse for substance abuse disorders.[28,29] Poor sleep quality has been shown to increase drug cravings,[41] exacerbate negative emotional states (associated with relapse),[32] and increase depressive symptoms.[27] Sleep disruption can also cause hyperalgesia (increased perception of pain), which leads to higher doses of opioids required for antinociception.[13,33] Differential effects of the three opioids on sleep are most likely due to pharmacokinetic and pharmacodynamic differences among these drugs, especially the half-life and potency.

Based on measures of 24-h sleep architecture and time-course of behavioral states following opioid administration reported in this study, buprenorphine caused longer sleep disruption compared to fentanyl or morphine before normalizing, relative to saline. This is likely related to the longer half-life of buprenorphine in comparison with morphine or fentanyl.[25] Results in this study also indicate that morphine caused a larger disruption in sleep and wakefulness (relative to saline) than fentanyl, especially during a 24-h recording. Morphine and fentanyl are both full mu-opioid receptor agonists; however, fentanyl is 50 to 100-times more potent, has a higher intrinsic efficacy at mu-opioid receptors, and a shorter half-life.[26] Morphine

causes a large increase in cortical histamine[42] and monoamines (such as histamine) have long been implicated in actively promoting wakefulness.[13]

Opioid-induced changes illustrated in this study created significantly different behavioral states based on physiological measures than normal states of sleep and wakefulness.[13,18] Mice in this study exhibited increased ambulation accompanied by the Straub effect[43] (a long-standing bioassay for morphine characterized by the tail pointed straight up in the air) following opioid administration. This finding is consistent with previous literature, which indicates that morphine and buprenorphine administered systemically to mice causes an increase in motor activity and in rats causes a loss of descending inhibition that is seen in the tail.[44] Disruptions in the sleep/wake cycle were illustrated qualitatively by EEG/EMG signals (Fig. 3) and graphs of 4-h sleep architecture following saline or opioid administration (Fig. 4). Further quantitative analysis elucidated disruption of major elements of healthy sleep architecture including: percent of time spent in states of sleep and wakefulness (Fig. 5 & 6), time course and light/dark phases of sleep (Fig. 7 & 8), number of episodes for each behavioral state (Fig. 9 & 10), duration of the episodes (Fig. 11 & 12), and the latency to onset of sleep (Fig. 13 & 14). These results collectively indicate that systemic opioid administration disrupts sleep/wake states in B6 mice.

Percent time in behavioral states was disrupted by opioids

Opioids caused an increase in the percent of time mice spent awake and decreased the percent of time spent in both NREM and REM sleep (Fig. 5 & 6). Morphine eliminated NREM sleep and buprenorphine eliminated both NREM and REM sleep in a 4-h recording (Fig. 5). The measure of sleep and wakefulness during control conditions of the present study were evaluated relative to previously published data describing the percent of time (mean ± SD) B6 mice

typically spend in states of wakefulness (59.4 ± 1.8), NREM sleep (35.6 ± 1.5) and REM sleep (4.8 ± 0.3) during a 24-h period.[45] Values reported in this study for 24-h sleep recordings following saline administration were within ± one standard deviation to those reported in previous literature. After injections of saline (control), NREM and REM sleep values were higher during the light cycle than during the dark cycle. This is in line with the physiology of these nocturnal animals. However even during the light phase, a time interval when these animals are typically sleeping, NREM and REM sleep were significantly decreased or eliminated by systemic opioid administration. Results from this study are consistent with data showing that patients receiving opioid treatments experience increased time awake and less overall sleep.[3,4] The present results from B6 mice are consistent with reports that morphine decreases NREM sleep in cats,[11] and decreases NREM and REM sleep in rats[9,10] and healthy humans.[13] Systemically administered buprenorphine increases time spent in wakefulness and decreases both NREM and REM sleep in rats.[8] Such findings suggest opioid-induced disruption of restorative sleep[13] and memory consolidation.[46] Altogether, the decrease in sleep following acute administration of fentanyl, morphine, or buprenorphine in this study is consistent with the hypothesis that systemically administered opioids disrupts sleep/wake states in B6 mice.

Opioids altered time course and light/dark cycle

The present study found that systemically administered fentanyl, morphine, and buprenorphine altered the time course (Fig. 7) of behavioral states during the light and dark periods (Fig. 8). After administration of fentanyl and morphine, sleep/wake states did not return to control levels for 4 h and 6 h, respectively. Fentanyl and buprenorphine caused sleep disruption lasting 4 h and 10 h post-injection with an increase in REM sleep at 14 h and 16 h,

respectively, during the dark cycle (Fig. 7). These data indicated that opioid administration caused significantly more time in wakefulness and less time in NREM and REM sleep during the light cycle when compared to saline. The REM sleep inhibition caused by fentanyl and buprenorphine was followed by a rebound increase in REM sleep during the dark cycle (Fig. 8). These results are of interest relative to previous studies indicating opioid use in humans is associated with increased daytime sleepiness,[5] poor daytime function,[1] and reduced nighttime sleep quality.[3,4]

Number of episodes was decreased by opioids

Opioids decreased the number of waking, NREM, and REM sleep episodes when compared to saline (Fig. 9 & 10). Following fentanyl, morphine, or buprenorphine administration, REM sleep episodes displayed a rebound increase during the dark cycle of the 24-h period (Fig. 10). This is likely due to decreased REM sleep episodes during the light cycle following opioid injection. Previous studies indicate opioid use causes abnormal sleep patterns[5,6] and disrupts multiple metrics of healthy sleep function.[3,13] The number of sleep/wake episodes is directly relevant as a functional metric of sleep health as a biological rhythm.[13,14,47]

The duration of individual sleep/wake episodes were disrupted by opioids

Opioids increased the duration of waking episodes and decreased the duration of both NREM and REM sleep episodes in a 4-h recording (Fig. 11). Recordings that were maintained for 24 h post-injection revealed that opioids disrupted the duration of both NREM and REM sleep episodes and illustrated that the trend is for opioids to increase the duration of waking episodes (Fig. 12). To facilitate the most conservative statistical analysis, data from all mice

were included in the Two-way ANOVA for the duration of waking episodes. Differences in sample sizes between treatment conditions dramatically increases the type-1 error rate for ANOVA by violating the assumption of equal variances.[48] The number of episodes for each measured behavioral state decreased during a 4-h recording, which resulted in more time spent in each state. This can also be understood as a hydraulic model (i.e., as one measure decreases, the other increases and vice versa) with the number of episodes. These findings are consistent with previous literature indicating opioid usage in humans is associated with longer periods of wakefulness and shorter sleep intervals.[7] Such effects in humans are important because the duration of sleep episodes (similar to number of episodes) is one measure of restful sleep.[13,14,47]

Opioids increased latency to onset of sleep

This study found that opioids increased the latency to onset of sleep (Fig. 13 & 14). Mice required significantly more time to enter states of NREM and REM sleep after opioid injection compared to saline. The differences in sample sizes between saline and opioid conditions for 4-h recordings based on elimination of NREM and REM sleep following opioid injection limited the ability to perform ANOVA.[48] This heterogeneity of variance required that non-parametric tests be used for this analysis. The Wilcoxon signed rank (non-parametric) test was utilized for analysis of 4-h recordings, because it compared an equal number of samples between saline and opioid treatment conditions in this model (i.e., 4 h recordings following injection saline versus fentanyl or morphine recordings, which contained NREM or REM sleep) and was unencumbered by the differences in sample size.[49] Buprenorphine administered systemically causes increased sleep latency in rats[8] and opioid use in humans is associated with increased sleep latency.[3,7] Delayed sleep latency is commonly associated with poor sleep quality and sleep disorders such as insomnia and narcolepsy.[50]

Limitations & Future Directions

Limitations of this study include use of only adult male mice, and it is well understood that there are gender and age differences in sleep quality and in sleep disorders.[51,52] This study chose to model clinical application of opioids by way of systemic drug delivery. The present results encourage future studies that use chronic administration of opioids. Sleep disturbance can increase over time during methadone treatment for OUD[7] and chronic opioid administration is common for management of cancer pain. The present study was also limited to studying the B6 line of mice. Another advantage of using mice is the availability of transgenic lines to model various diseases. The present results encourage the use of obese mice that lack leptin or have disrupted leptin receptors. In addition to functioning as a satiety factor, leptin status alters the antinociceptive effects of opioids.[53] Finally, the present results also encourage future studies that aim to evaluate dose-response relationships for the effects of fentanyl, morphine, and buprenorphine on NREM and REM sleep.

Despite the foregoing limitations, the present findings provide novel evidence that opioids cause sleep disruption in B6 mice that are similar to the sleep disruption caused by opioids in humans. These similar opioid effects are consistent with the close homology between the human and B6 mouse genome. Considered together, the results support the likelihood that studies of opioid effects using the B6 mouse will help provide a better understanding of the mechanisms underlying opioid-induced sleep disruption in humans.

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Vita

Clarence Earl Locklear was born in Durham, North Carolina to Sarlenna and Robert Locklear. Hehas three siblings: RJ, Kya, and Haven. He moved to Greeneville, Tennessee at the age of 11. He graduated from Greeneville High School in 2011. He graduated with his B.A. in Psychology from the Univeristy of Tennessee, Knoxville in Decemeber 2015. He applied to graduate school at the Universtiy of Tennessee for a Masters in Experimental Biological Psychology under the mentorship of Dr. Ralph Lydic and was fortunate to be accepted in Fall 2018. He plans to complete his degree in July of 2020. After graduation, Clarence plans to continue his career in academia by pursuing a Ph.D in Pharmacology and Neuroscience at the Univeristy of Miami, Miller School of Medicine beginning Fall 2020. Clarence is a registered member with the Lumbee tribe of North Carolina. In his free time, he enjoys training in Jiu-jitsu and hiking with his German Shepherd dog, Nova.