Opioid-induced sleep disruption in C57BL/6J mice: A systems physiology perspective

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Diana Zebadua Unzaga

May 2025

Dedication

This dissertation is dedicated to my husband Matt and my children Aaron and Maia, for their support, patience and constant love that pushed me to go further, even on the days when it felt like I had nothing else to give.

To my mom Silvia, my dad Chava (from wherever you are), my brother Daniel, my cousin and sister Leslie, and all my family - for their love, constant support, and unwavering belief in me since I was a little kid, and for giving me the tools to get to where I am today.

To my friends, my chosen family, who held my hand so many times. Thank you for your love and support, the lunches, phone calls, Zoom calls, texts and memes have all been essential for keeping me going.

To my mentors, Dr. Helen A. Baghdoyan and Dr. Ralph Lydic without whom I could not have reached this point. I feel so fortunate to have found such incredible people, scientists, and mentors. The support and guidance that you gave me always went beyond the call of duty. I truly could not have done this without you. My family and I are immensely grateful, and we consider you now my family away from home.

Each and every one of you has had such a profound impact on my life, and I want you to know that this is not just my accomplishment; it is also yours. Thank you for believing in me. Thank you from the bottom of my heart.

Acknowledgements

Before starting a doctoral program, one might imagine that it will require the support of many people to achieve a successful outcome. Well, that is not far at all from the truth, at least in my case. Without the support and collaboration of many individuals, this dissertation would not have been possible.

Although the people mentioned here are not listed in a particular order, I want to begin by expressing my gratitude to my mentors, Dr. Baghdoyan and Dr. Lydic. Your love for science, passion for learning and teaching, dedication, commitment to the highest standards, your willingness to help even the most challenging students have shown me that it is possible excel professionally while remaining compassionate and humble. My time in your lab was, in every positive sense, life- and career-altering. I look forward to what comes next and hope to continue collaborating with you in the future.

To my dissertation committee members, Dr. Daniel Jacobson and Dr. Reza Seddighi, thank you for your thoughtful comments and insightful suggestions throughout this process. Your time and expertise pushed me to go beyond my comfort zone and have been instrumental in helping me become a better scientist.

To my fellow graduate colleagues and undergraduate students in the lab, I am deeply grateful for your hard work and dedication. Zach Glovak, Chris O'Brien, Clarence Locklear, Daniel Woods, and Natania Cooper, your unique perspectives and knowledge not only expanded my own knowledge, but also taught me to approach things from different angles. I learned so much from each one of you, and I want you to know that you do not just have a colleague in me, you also have a friend. To the many undergraduate students whose dedicated efforts and countless hours of double-scoring were indispensable to this dissertation, thank you. You enriched this

experience in every way.

Thank you to the Psychology Department for your commitment to supporting students. My graduate experience would not have been possible without your support and unmeasurable efforts to meet the unique needs of each student. A special thank you to Connie Ogle, your dedication and hard work reveal how much you love what you do. Words cannot fully express my gratitude for the immense support you provide to each of us.

To my in-laws, Gail and Gene, and my mother, without whom I would not have been able to complete my degree. Childcare is not easy to arrange, but what a blessing to have those you love and trust care for your children when you are in need. Thank you from the bottom of my heart.

Finally, one more time, to my ultimate support team, the ones that I relied on day and night. My family: Matt, Aaron and Maia, for unconditional love, unshakeable support and constant encouragement. For believing in me every step of the way, this one is for you, with all my love. Thank you!

Abstract

This dissertation was motivated by the ongoing opioid epidemic and the substantial impact of opioids on sleep health. While opioids are well-documented sleep disruptors, their effects on sleep have been understudied. Sleep plays a key role in overall health, and is closely interrelated to many other physiological systems, including motor activity control and body temperature regulation.

The chapters of this dissertation investigate the dose-dependent effects of morphine and fentanyl on sleep amount and architecture. Given that sleep homeostasis is a tightly regulated and multifactorial process, this work also examines the dose-dependent effects of opioids on EEG power, motor activity, and body temperature. Although the dependent variables observed here have been studied previously, they are often studied independently. This dissertation adopts an integrative approach to investigate the interaction among the effects of opioids on sleep, motor activity, and body temperature. To address these objectives, male B6 mice were used to simultaneously record sleep/wake states, motor activity and subcutaneous body temperature. The interactions among the dependent variables were evaluated through mediation analysis, a statistical approach based on structural equation modeling.

Chapters 2 and 3 detail the effects of antinociceptive doses of opioids on sleep and EEG. Chapter 3 expands on this by exploring the dose-dependent effects and depicting four-point logarithmic dose-response curves of the effects of fentanyl and morphine on sleep, EEG power, motor activity and body temperature.

The findings presented in this dissertation offer novel insights into the mechanisms underlying opioid-induced sleep disruption, suggesting that in B6 mice this disruption is at least partially secondary to the opioid-induced increases in motor activity. Taken together, this work provides

a new perspective through an integrative approach commonly used in systems physiology. The results shown here also underscore the importance of evaluating together opioid-induced adverse effects to better inform new strategies for the management of opioid-induced sleep disruption, pain management, addiction and overall opioid use.

Table of Contents

List of Tables

List of Figures

Chapter 1 : Introduction

Opioids and Sleep: Societal relevance and risks.

The goal of this introduction is to provide a context for research described in this dissertation. There is a long history of confusion regarding sleep and opioids. The term opioids refers to a large class of medications that share a chemical structure that is similar to the plant alkaloids derived from the opium poppy (Papaver somniferum) (Vadhel et al., 2023). Opium consists of various secondary constituents such as morphine, codeine, papaverine, and noscapine (Hayat et al., 2023; Morimoto et al., 2001; Wheeler, 2019). Alkaloids are secondary metabolites present in higher plants that have important functions in plant growth, morphology and development (Mohnen, 2008; Morimoto et al., 2001). Plant alkaloid concentrations increase with stress and serve as a defense mechanism against herbivores and microorganisms such as viruses, fungi and bacteria (Wink, 2018; Yeshi et al., 2022). Opiates derived from the opium produced by the plant are potent modulators of specialized G-protein-coupled receptors (GPCRs) that are largely inhibitory in metabolic, developmental and behavioral functions (Karnib et al., 2023). One function regulated by GPCRs is hunger. It has been hypothesized that plants developed alkaloids to increase satiety in herbivores that intend to feed on the plant's seeds, therefore reducing the damage that herbivores cause to the plants and ensuring survival (Karnib et al., 2023; Wink, 2018).

Drosophila express GPCRs that appear to have evolved from common ancestors with mammals. Moreover, there is evidence that *Drosophila*, similar to mammals, are sensitive to the locomotor and feeding effects of opioids (Hewes & Taghert, 2001; Karnib et al., 2023). Poppies evolved to produce opium as a defense mechanism. The similarity of GPCRs between mammals and insects renders mammals susceptible to these compounds.

Opioids have been used for thousands of years and across many cultures to relieve pain

(Brownstein, 1993; Vadhel et al., 2023). The Latin genus and species (binomial) names translate in English to “sleep-bringing poppy”. This misnomer came into use hundreds of years before neuroscience demonstrated that in all mammals, opioids produce a diminished level of electroencephalographic (EEG) and behavioral arousal while disrupting the temporal organization of sleep (de Andres et al., 1984; Greenwald & Roehrs, 2005; Lutz & Kieffer, 2013; Osman et al., 2005; Pickworth & Sharpe, 1979). The naming of Papaver somniferum is clear to anyone who has personally experienced or worked to help diminish extreme pain in another organism. In human and non-human animals, extreme pain is sometimes described as “writhing pain”. Administering opioids in such cases can decrease pain and quiet the vocalization and body contortions evoked by extreme pain. Also relevant to understanding the inappropriate description of opioids as causing somniferum is that opioids have been used for more than 1000 years BCE to treat pain (Brownstein, 1993). In contrast, electrophysiologically identified states of sleep were reported in 1953 (Aserinsky & Kleitman, 1953). The foregoing explains, in part, how the confusion between the obtunded state produced by opioids and physiological sleep arose.

Opioids act on endogenous opioid receptors which are comprised of three major families, mu, delta, and kappa. Different opioids bind either fully or partially to these receptors. The studies described in this dissertation provide novel quantification of sleep disruption in mice caused by the mu opioid receptor agonists morphine and fentanyl.

Morphine is one of the oldest and most used opioids in medical care. Morphine is considered a prototype or gold standard opioid and is commonly used clinically to estimate analgesic and adverse effects through an overall daily opioid-dose equivalency (Bhatnagar & Pruskowski, 2022). This tool for managing the dose and timing of opioid administration across many species

is referred to as morphine milligram equivalents, or MME (www.cdc.gov/drugoverdose/training/dosing/accessible/index.html). Fentanyl is a synthetic opioid that is fast acting, highly potent (50- to 300-fold more potent than morphine), and one of the most widely used drugs for pain management (Joranson et al., 2000).

Multimodal analgesia is also used to help clinicians pharmacologically manage pain. Nonetheless, opioids remain one of the main and most powerful tools for pain management (Benyamin et al., 2008; Bhatia & Buvanendran, 2019), despite the known adverse effects of tolerance and the potential for physical dependence and addiction (Benyamin et al., 2008).

The high opioid prescription rate in the United States has contributed to an epidemic of misuse, addiction, and overdose deaths (National Center for Drug Abuse Statistics, 2023; Rummans et al., 2018; Volkow & McLellan, 2016) that has developed into a public health crisis (Srivastava & Gold, 2018). During 2019 while in the middle of an opioid epidemic, the COVID-19 pandemic worsened the opioid epidemic. The COVID-19 pandemic was estimated to increase the total deaths from opioid overdose by 31% from 2019 to 2020 (Ghose et al., 2022; Hedegaard et al., 2021).

Sleep

Sleep is a reversible behavioral state during which the subject is disengaged from and unresponsive to external surroundings (Carskadon & Dement, 2011). Sleep in vertebrate mammals is manifest as two distinct states comprised of different physiological and behavioral traits. The distinct sleep states are referred to as rapid eye movement (REM) sleep, named for the physiological trait of eye movements, and non-REM (NREM) sleep. Given that both NREM and REM sleep exist in virtually all mammals and birds it can inferred that sleep plays a vital role in the maintenance of health (Siegel, 2008).

My previous training in veterinary medicine gives me an appreciation of the fact that states of sleep are widely observed across animal phyla (Bódizs et al., 2020; Gaviraghi Mussoi et al., 2022; Greening et al., 2021). Dream reports recorded in the Egyptian Book of the Dead (Andrews, 1985) indicate that humans of 3,000 years ago understood in some way that perceptions during sleep were very different than during wakefulness. In 1957 it was discovered that REM sleep also occurs in non-human animals as a distinct physiological state (Dement & Kleitman, 1957). Human sleep disorders have likely been present throughout the epoch of modern homo sapiens but was it not until 1993 that the U.S. National Institutes of Health included a Center for Sleep Medicine (Shepard Jr et al., 2005).

Currently, there is a nosology listing 81 sleep specific disorders (Sateia & Thorpy, 2017). An estimate of 50 to 70 million people in the United States chronically suffer from some kind of sleep disorder, which adversely affects the daily lives, health, and longevity of these individuals (Weir, 2022). Normal sleep is disrupted by pain and approximately 51.6 million people in the U.S. experience chronic pain (Rikard et al., 2023). As reviewed recently (Lydic et al., 2022), the relevance of the association between sleep and opioid use also relates to the fact that there is a bi-directional relationship between sleep disruption and addiction (Tripathi et al., 2020). Sleep disruption is considered one of the risk factors for relapse in people recovering from addiction to psychoactive substances (Brower & Perron, 2010; Moore & Dimsdale, 2002). Furthermore, withdrawal from addiction to a range of drugs is associated with severe sleep disruption (Brower & Perron, 2010; López-Muciño et al., 2022; Reid-Varley et al., 2020). Additionally, decreased sleep promotes hyperalgesia and lowers pain thresholds, thereby perpetuating a vicious cycle. The research presented in this dissertation quantifying the effects of opiates on sleep is therefore also relevant to substance abuse disorder.

Using mice to advance understanding of adverse effects of opioids.

Preclinical biomedical studies using mice make common use of the phrase "mouse models" reflecting an intent to derive information relevant to human health. This is an important goal for publicly funded research of course, but "mouse model" can be misread as illustrated by an opinion piece from the Editor-in-Chief of the Journal of the American Association for Laboratory Animal Science. That editorial raised the question for investigators to consider: "Laboratory animals, animal models, or just animals?" (Toth, 2023). Companion animals, our dogs or cats, are not merely "dog models" or "cat models" of loyal and affectionate beings. One alternative is the now established evolutionary connection between all vertebrate mammals as reflected in the One Health concept (Evans & Leighton, 2014). The fact of ongoing climate change illustrates that the One Health model is also a valuable tool for disease prediction (Rabinowitz et al., 2013) and food security (King, 2012).

Additional justifications for the use of mice in the present series of experiments are provided by genetics and ethics. The C57BL/6J (B6) mice used in this dissertation (**Figure 1.1**) were derived in the 1920s from a selective breeding program by Clarence Cook Little at the Jackson Laboratory (Spencer, 2012). The initial sequencing of the B6 mouse genome was published in December 2002 issue of Nature (Consortium, 2002). The B6 genome has an approximately 96% homology to the human euchromatic sequence, encouraging the use of B6 mice for One Health-related research (Benyamin et al., 2008; Chen et al., 2021; Consortium, 2002; Gordon, 2017; Gunter, 2003).

From an ethical perspective the studies reported in this dissertation can only be conducted using non-human animals. The independent variables used in the present study were eight concentrations of morphine and fentanyl systemically administered to mice before obtaining 4-h

recordings of electroencephalogram (EEG), electromyogram (EMG), motor activity, and body temperature that were then compared to 4-h recordings after saline (control) injections. Morphine and fentanyl each have high abuse potential and a sigmoidal relationship between respiratory depression and the concentration of administered opioid. All experiments reported in this dissertation were reviewed and approved by The University of Tennessee, Institutional Animal Care and Use Committee (IACUC).

The following subsections of this Introduction focus on each of the four dependent measures quantified by this dissertation research. These sections include morphine and fentanyl-induced alterations in 1) sleep wake states, 2) EEG frequency and power, control of 3) movement and 4) body temperature. The goal of each subsection is to provide a brief historical and conceptual context for the dependent measures reported in this dissertation. After this overview, the remaining chapters of this dissertation include detailed analyses comprising the published results and the results currently undergoing pre-publication peer review.

Mouse studies of sleep wake states before and after receiving opioids

Measures of the human electroencephalogram (EEG) across the sleep/wake cycle began in 1935 (Loomis et al., 1935). Continuing advances in microelectronics eventually made it possible to record the EEG of mice across the sleep/wake cycle. These technical advances made it possible for my research to objectively quantify states of wakefulness, NREM sleep, and REM sleep via recordings of EEG, EMG, motor activity, and body temperature. This was accomplished by linking the EEG and EMG electrodes to a subcutaneously implanted telemeter which also recorded body temperature and mouse movement. Additionally, the implanted telemeter signaled to an antenna placed under the mouse home cage which enabled studies of freely behaving mice (see Fig. 1.1) not encumbered by a wire cable attached to an electrode headcap.

To establish mice as a viable model for sleep research, NREM sleep and REM sleep had to be characterized in these nocturnal rodents (Valatx & Bugat, 1974). Those studies and others, conducted across the 12:12 light:dark cycle determined that predominant sleep activity in mice occurs during the light phase but some episodes of NREM and REM sleep occur during the dark phase (Mitler et al., 1977). Additionally, it was observed that tongue movements in mice during REM sleep can be considered analogous to ocular movements in humans (Faradji et al., 1980). Similar to humans, mice also express a ~24 h circadian pattern that modulates the physiological responses and sensitivity to drugs such as morphine (Oliverio et al., 1982). This intrinsic circadian rhythm, along with the drive for sleep provided evidence that NREM sleep, REM sleep, and wakefulness activity comprise an endogenous biological rhythm (Richardson et al., 1985; Welsh et al., 1986). These early studies presaged the 2017 Nobel Prize winning discoveries of the molecular biology underlying circadian biology (Huang, 2018). Further studies in mice have explored genetic (Franken et al., 1999) and age-dependent (Panagiotou et al., 2017) factors that affect states of sleep and the traits characteristics of NREM and REM sleep. Such findings serve to strengthen the statement that although mice are nocturnal animals, the resemblance of their sleep patterns to those observed in humans renders them a valuable model for the study of sleep-related research.

Mice long have been used to investigate the impact of opioids on sleep and have been key to confirm the role of mu receptors in the effect of morphine on sleep (Eacret et al., 2022), pain, and physical dependence (Becker et al., 2000; Matthes et al., 1996). The present derivation of opioid dose-response curves in mice was an essential step for opioid research seeking a more detailed understanding of pharmacology, antinociceptive actions, and changes in serum and brain tissue concentrations of opioids (Kalvass et al., 2007).

Studies of opioid actions across the sleep/wake cycle have established that opioids disrupt sleep (Cronin et al., 1995; Lydic et al., 2022) by decreasing NREM sleep and suppressing REM sleep in humans (Dimsdale et al., 2007; Lydic et al., 2017), rats (Gauthier et al., 2011; Montandon & Horner, 2019), and mice (Zebadúa Unzaga et al., 2021). Additionally opioids have been found to prolong the latency to sleep onset (Mehtry et al., 2014) and disrupt sleep architecture by inducing sleep fragmentation in humans (Greenwald et al., 2021). As part of this dissertation research, antinociceptive doses of morphine, fentanyl, and buprenorphine were shown to significantly decrease EEG power at specific frequency bands and disrupt the normal temporal organization of states of wakefulness, NREM sleep, and REM sleep (O'Brien et al., 2021). The results of my dissertation research add novel findings concerning the dose-dependent effects of morphine and fentanyl on opioid naïve, freely behaving B6 mice. Measures of sleep quantified the amount of sleep and temporal organization of states of wakefulness, NREM sleep and REM sleep. The results reported in this dissertation support and expand upon my preliminary findings (Zebadúa Unzaga et al., 2021; Zebadúa Unzaga et al., 2023; Zebadúa Unzaga et al., 2022).

Opioid-induced alterations of EEG frequency and power in B6 mice

For many decades, there was a lack of electronic circuits that could selectively record different electroencephalogram (EEG) frequencies. During the development of basic and clinical neuroscience, the recordings of EEG have provided an objective measure of sleep/wake states. EEG recordings have shown that mice (Valatx et al., 1972) homologous to humans, have qualitative and quantitative differences in EEG patterns that change depending on the states of consciousness (Adamantidis et al., 2019; Herrmann et al., 2016).

EEG studies have also enabled standardization and naming of EEG frequencies (Nuwer, 1988). This standardization has been used to selectively evaluate the effect of opioids on frequencies

(Hz) corresponding the following nomenclature: delta (0.5 to 4Hz); theta (4 to 8Hz); alpha (8 to 12Hz); sigma (12 to 16Hz) and beta (13 to 30Hz) (Nayak & Anilkumar, 2024; Prerau et al., 2017). Using that nomenclature, studies have provided mouse age (Panagiotou et al., 2017), strain (Franken et al., 1998, 1999; Siegfried et al., 1980), and brain region-specific (Huber et al., 2000) descriptions of the EEG. The descriptions show noticeable state-specific EEG power variations observed consistently across all mammals. These include high theta power dominating exploratory behavior during wakefulness (Franken et al., 1998; Scheffzük et al., 2011; Welsh et al., 1985), increased delta power during SWS and increased theta power during REM sleep (Franken et al., 1998, 1999; Welsh et al., 1985).

My research on the EEG effects of opioids on mice found that fentanyl administered during wakefulness increased delta power and reduced theta, alpha, and beta power, thus creating dissociated states of consciousness (O'Brien et al., 2021). Studies of male Wistar rats have found positive correlations between fentanyl-induced respiratory depression, high EEG theta power (Montandon & Horner, 2019), and elevated delta power with increased neuropathic pain (Li et al., 2020). EEG power long has been known to be altered by opioids. However, the studies comprising this dissertation quantified, for the first time in B6 mice, the dose-dependent nature of EEG changes caused by fentanyl and morphine.

Opioid-induced changes in mouse motor activity

For decades opioids have been used for pain management. However, these medications can disrupt motor control, leading to undesired effects such as gastrointestinal smooth muscle depression, which can result in ileus and constipation (Kurz & Sessler, 2003). With the ability to record bioelectric potential of nerves, neurons, and muscle units it became clear that opioids hyperpolarize neurons resulting in the desired effects of anti-nociception, and undesired effects

of electromyographic (EMG) activity reduction in the upper airway (Freire et al., 2022; Hajiha et al., 2009) intercostal (Wanke et al., 1993) and diaphragmatic muscles (Torralva & Janowsky, 2019). Respiratory depression or hypoventilation is without a doubt the most concerning adverse effect of opioids and the leading cause of opioid misuse deaths (Bateman et al., 2021; Freire et al., 2022). Considering that the effects of opioids on breathing are related to the motor control of respiration, evaluation of the effect of opioids on motor activity is essential.

The effect of opioids on motor activity in mice has long been recognized (Goldstein & Sheehan, 1969; Haouzi et al., 2021). Initially, locomotion was used as a gauge for assessing the effects of opioids in mice (Goldstein & Sheehan, 1969). Research through the years has demonstrated that opioid-induced motor activity changes are species (Kuschinsky & Hornykiewicz, 1974), strain (Judson & Goldstein, 1978; Oliverio & Castellano, 1974), age (Hofford et al., 2012; Hoskins et al., 1986; Koek, 2014), and sex dependent (Hámor et al., 2023).

In mice, unlike humans, the opioid-induced effects on locomotor activity are excitatory. The hyperlocomotion pattern has been described as "continuous, unidirectional and thigmotactic" (Santos et al., 2022). In mice, the thigmotactic response refers to mice avoiding the open region of an enclosure and exhibiting a preference for the periphery of any environment. Opioid effects on motor activity have been shown to be drug, dose, and time specific. A brief report from 1980 noted that in CFLP mice morphine caused a biphasic effect on locomotion with an initial locomotor depression followed by hyperactivity (Miglécz & Rónai, 1980). The initial decrease in motor activity occurred at lower doses of 3 mg/kg of morphine and was followed by an increase in motor activity (Miglécz & Rónai, 1980; Patti et al., 2005; Zebadúa Unzaga et al., 2023). Higher doses (10 - 30 mg/kg) of morphine increased motor activity beginning within 10 min after drug administration (Patti et al., 2005; Zebadúa Unzaga et al., 2023). In rats (Gaulden et al.,

2021; Zhang & Kong, 2017) and mice (Varshneya et al., 2019, 2021) increased locomotor activity has been observed for morphine, fentanyl, and several fentanyl-related substances. The effect of opioids on motor activity in mice usually reaches a peak shortly after administration, with a duration of about 2 h, then returns gradually to baseline (Kitanaka et al., 2023).

Although the mechanisms by which opioids alter motor activity are incompletely understood, motor control is known to involve multiple neuronal networks. For example, opioid activation of locomotion involves enhancement of the mesolimbic dopamine system (Santos et al., 2022). The mesolimbic structures are among the areas with the highest opioid receptor concentration and have also been shown to regulate motor functions (Pert et al., 1979; Serafini et al., 2020). In contrast to opioid effects in mice, humans administered opioids do not commonly show an increase in locomotor activity. As noted above, opioids administered to humans cause states of blunted wakefulness characterized as drowsiness and/ or lethargy with decreased motor activity (Benyamin et al., 2008; Paul et al., 2021; Raffaeli et al., 2006). This species-specific difference in motor activity caused by opioids encouraged me to quantify the extent to which morphine and fentanyl induced sleep disruption is caused by increased locomotor activity.

Opioid-induced changes in subcutaneous body temperature

Body temperature is one of the most tightly regulated parameters among physiological processes. Although an in-depth discussion of thermoregulation is not the scope of this dissertation, some background on temperature regulation and its relationship to sleep is necessary to understand how opioids alter the relationship between body temperature and sleep/wake states.

Body temperature regulation involves multiple mechanisms to maintain homeostasis by balancing heat production and heat dissipation. Mammals maintain a nearly constant core body temperature based on a target single reference value that the body tries to preserve to achieve

optimal function. The target reference value is called a set point and does not change more than a few tenths of a degree (Romanovsky, 2018). However, slight variations in the set point can be expected due to the circadian rhythm and cyclic hormonal variations (Kurz, 2008). Behaviors such as feeding and energy expenditure are important aspects of thermoregulation (Belknap, 1989; Cintron-Colon et al., 2019). In healthy mammals the relationship between sleep and body temperature can be observed during NREM sleep, when core body temperature is lower than during wakefulness. A decrease in body temperature is often considered a signal for the onset of NREM sleep (Harding et al., 2019; Harding et al., 2020; Heller, 1988). During REM sleep in rodents body temperature is not regulated (Harding et al., 2020), thus body temperature is highly sensitive to ambient temperature. Lower room temperatures decrease the occurrence of REM sleep bouts. Therefore, in order to have restorative REM sleep, thermoneutrality is needed (Amici et al., 2008; Harding et al., 2019). Thermoneutrality is the state in which the ambient temperature does not require an organism to lose or gain heat (Gordon, 2012). In humans, temperature changes are so important for sleep that the fluctuations in temperature that normally occur prior to sleep onset synchronize with our daily rhythms. This means that there is a change in body temperature when sleep is expected or needed, even if sleep is delayed (Gilbert et al., 2004). Any condition that affects temperature regulation can also affect the ability to initiate or maintain sleep and can alter sleep architecture (Okamoto-Mizuno & Mizuno, 2012). Sleep/wake states are considered the primary drivers of temperature regulation, whereas circadian phase and physical activity are secondary drivers (Harding et al., 2020).

Changes in body temperature can also be drug induced. Typically, these changes result from a shift in the hypothalamic regulated temperature set point (Belknap, 1989). When a drug changes the set point, the body still maintains temperature within a narrow range. Conversely, if the drug

alters thermoregulatory mechanisms such as feeding, shivering, or sweating, then the drug is impairing or diminishing the body's ability to maintain the set temperature by altering the balance between heat production and dissipation (Adler et al., 1988).

Opioids have long been known to alter mammalian thermoregulation. The endogenous opioid system serves several physiological functions in the body, including thermoregulation (Kozak et al., 1995; Rosow et al., 1980). Consequently, exogenous opioid administration can cause temperature changes such as hypo- and hyperthermia in mice (Baker & Meert, 2002; Belknap, 1989; Zebadúa Unzaga et al., 2023), humans (Ikeda et al., 1997), and rats (Pert et al., 1979; Savić Vujović et al., 2013). The available data indicate that although all opioid-induced changes in body temperature have a similar trend to cause hypothermia, variations are dependent on the species (Gaulden et al., 2021; Ikeda et al., 1997; Rosow et al., 1980), strain (Rawls & Benamar, 2011), sex (Craft, 2003; Kest et al., 2000), age (Koek et al., 2012), environmental temperature (Sadowski et al., 1996), dose (Geller et al., 1983; Rosow et al., 1982), and the specific opioid used (Baker & Meert, 2002).

The anteroventral preoptic region (POA) in the hypothalamus is key in the regulation of heat production and dissipation. The POA controls thermoregulatory mechanisms such as vasodilation and brown adipose tissue thermogenesis (Rothhaas & Chung, 2021). Ther foregoing data suggest that opioid-induced decreases in body temperature are at least partially mediated by mu opioid receptors located in the POA (da Silveira Scarpellini et al., 2009).

In adult humans, opioids affect thermoregulatory mechanisms by increasing sweating, decreasing vasoconstriction, and decreasing shivering (Ikeda et al., 1997) . These effects can lead to hypothermia and cause awakenings or difficulty staying asleep. These awakenings lead to fragmented sleep or sleep that is not restorative, therefore reducing the sleep efficiency and

quality (Stepanski, 2002; Wesensten et al., 1999).

Despite numerous studies examining the effect of opioids on body temperature, the specific mechanisms by which opioids alter thermoregulation and the impact of said temperature changes on sleep remain incompletely understood. This gap in the literature encouraged the present studies designed to quantify the extent to which the morphine- and fentanyl-induced hypo- and hyperthermia causes sleep disruption.

Summary

Opioids are a well-established treatment for pain. However, the benefits of opioids are accompanied by several adverse effects (Benyamin et al., 2008; Bhatia & Buvanendran, 2019) that include sleep disruption. The homology and similarity of sleep patterns of the B6 mouse to humans (Consortium, 2002) make the B6 mice a valuable model for the study of sleep-related effects of opioids.

Normal sleep has been shown to be disrupted by pain (Finan et al., 2013) and it is now clearer than ever that there is a bidirectional relationship between sleep disruption and addiction (Brower & Perron, 2010; Lydic et al., 2022; Tripathi et al., 2020). Disturbed sleep decreases pain threshold, patient increases opioid intake, worsening sleep and consequently pain, therefore creating a vicious cycle that increases the probability of addiction (Roehrs et al., 2021).

Sleep is intrinsically related to other physiological processes such as EEG power, motor activity and body temperature. The studies completed for this dissertation present novel data from mice quantifying the dose-dependent effects of morphine and fentanyl on the amount and temporal organization of states of wakefulness, NREM sleep and REM sleep. The research presented here also shows new data simultaneously quantifying, for the first time, the dose-dependent effects of fentanyl and morphine on EEG power, motor activity, and subcutaneous body temperature.

Additionally, this dissertation explores the relationship between sleep and opioid-induced altered states of consciousness, and considers interactions among sleep, motor activity and body temperature.

References

Adamantidis, A. R., Gutierrez Herrera, C., & Gent, T. C. (2019). Oscillating circuitries in the sleeping brain. *Nature Reviews Neuroscience, 20*(12): 746-762. https://doi.org/10.1038/s41583-019-0223-4

Adler, M. W., Geller, E. B., Rosow, C. E., & Cochin, J. (1988). The opioid system and temperature regulation. *Annual review of pharmacology, 28*(1): 429-449. https://doi.org/10.1146/annurev.pa.28.040188.002241

Amici, R., Cerri, M., Ocampo-Garcés, A., Baracchi, F., Dentico, D., Jones, C. A., Luppi, M., Perez, E., Parmeggiani, P. L., & Zamboni, G. (2008). Cold exposure and sleep in the rat: Rem sleep homeostasis and body size. *Sleep, 31*(5): 708-715. https://doi.org/10.1093/sleep/31.5.708

Andrews, C. (1985). *The ancient egyptian book of the dead* (R. O. Faulkner, Trans.). New York : Macmillan, 1985. https://search.library.wisc.edu/catalog/999631563402121

Aserinsky, E., & Kleitman, N. (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. *Science, 118*(3062): 273-274. http://www.jstor.org.utk.idm.oclc.org/stable/1680525

Baker, A. K., & Meert, T. F. (2002). Functional effects of systemically administered agonists and antagonists of μ, δ, and κ opioid receptor subtypes on body temperature in mice. *The Journal of Pharmacology and Experimental Therapeutics, 302*(3): 1253-1264. https://doi.org/10.1124/jpet.102.037655

Bateman, J. T., Saunders, S. E., & Levitt, E. S. (2021). Understanding and countering opioid-induced respiratory depression. *British Journal of Pharmacology, 180*(7): 813-828. https://doi.org/10.1111/bph.15580

Becker, A., Grecksch, G., Brödemann, R., Kraus, J., Peters, B., Schroeder, H., Thiemann, W., Loh, H. H., & Höllt, V. (2000). Morphine self-administration in μ-opioid receptor-deficient mice. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *361*(6): 584-589. https://doi.org/10.1007/s002100000244

Belknap, J. K. (1989). Components of the opioid withdrawal syndrome in mice are thermoregulatory responses. *Pharmacology Biochemistry and Behavior*, *34*(2): 241-245. https://doi.org/10.1016/0091-3057(89)90306-7

Benyamin, R., Trescot, A. M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., Glaser, S. E., & Vallejo, R. (2008). Opioid complications and side effects. *Pain Physician*, *11*(2 Suppl): S105-120. https://doi.org/10.36076/ppj.2008/11/S105

Bhatia, A., & Buvanendran, A. (2019). Anesthesia and postoperative pain control-multimodal anesthesia protocol. *J Spine Surg*, *5*(Suppl 2): S160-s165. https://doi.org/10.21037/jss.2019.09.33

Bhatnagar, M., & Pruskowski, J. (2022). *Opioid equivalency* (T. I. (FL), Ed.). StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC. https://www.ncbi.nlm.nih.gov/books/NBK535402/

Bódizs, R., Kis, A., Gácsi, M., & Topál, J. (2020). Sleep in the dog: Comparative, behavioral and translational relevance. *Current Opinion in Behavioral Sciences*, *33*: 25-33. https://doi.org/10.1016/j.cobeha.2019.12.006

Brower, K. J., & Perron, B. E. (2010). Sleep disturbance as a universal risk factor for relapse in addictions to psychoactive substances. *Medical Hypotheses*, *74*(5): 928-933. https://doi.org/10.1016/j.mehy.2009.10.020

Brownstein, M. J. (1993). A brief history of opiates, opioid peptides, and opioid receptors.

Proceedings of the National Academy of Sciences, *90*(12): 5391-5393. https://doi.org/10.1073/pnas.90.12.5391

Carskadon, M. A., & Dement, W. C. (2011). Chapter 2 - normal human sleep: An overview. In M. H. Kryger, T. Roth, & W. C. Dement (Eds.), *Principles and practice of sleep medicine (fifth edition)* (pp. 16-26). W.B. Saunders. https://doi.org/10.1016/B978-1-4160-6645-3.00002-5

Chen, C.-H., Liao, C.-H., Chen, K.-C., Wang, K.-L., Tseng, X.-W., Tsai, W.-K., Chiang, H.-S., & Wu, Y.-N. (2021). B6 mouse strain: The best fit for lps-induced interstitial cystitis model. *International Journal of Molecular Sciences*, *22*(21): 12053. https://doi.org/10.3390/ijms222112053

Cintron-Colon, R., Johnson, C. W., Montenegro-Burke, J. R., Guijas, C., Faulhaber, L., Sanchez-Alavez, M., Aguirre, C. A., Shankar, K., Singh, M., Galmozzi, A., Siuzdak, G., Saez, E., & Conti, B. (2019). Activation of kappa opioid receptor regulates the hypothermic response to calorie restriction and limits body weight loss. *Current Biology*, *29*(24): 4291-4299.e4294. https://doi.org/10.1016/j.cub.2019.10.027

Consortium, M. G. S. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, *420*(6915): 520-562. https://doi.org/10.1038/nature01262

Craft, R. M. (2003). Sex differences in opioid analgesia: "From mouse to man". *The Clinical Journal of Pain*, *19*(3): 175 - 186. https://doi.org/10.1097/00002508-200305000-00005

Cronin, A. J., Keifer, J. C., Baghdoyan, H. A., & Lydic, A. R. (1995). Opioid inhibition of rapid eye movement sleep by a specific mu receptor agonist. *British Journal of Anaesthesia*, *74*(2): 188-192. https://doi.org/10.1093/bja/74.2.188

da Silveira Scarpellini, C., Gargaglioni, L. H., Branco, L. G. S., & Bícego, K. C. (2009). Role of

preoptic opioid receptors in the body temperature reduction during hypoxia. *Brain Research, 1286*: 66-74. https://doi.org/10.1016/j.brainres.2009.06.039

de Andres, I., Villablanca, J. R., & Burgess, J. W. (1984). Reassessing morphine effects in cats: Ii. Protracted effects on sleep-wakefulness and the eeg. *Pharmacology Biochemistry and Behavior, 21*(6): 923-928. https://doi.org/10.1016/S0091-3057(84)80074-X

Dement, W. C., & Kleitman, N. (1957). Cyclic variations in eeg during sleep and their relation to eye movements, body motility and dreaming. *EEG Clin Neurophysiol, 9*: 673-690. https://doi.org/10.1016/0013-4694(57)90088-3

Dimsdale, J. E., Norman, D., DeJardin, D., & Wallace, M. S. (2007). The effect of opioids on sleep architecture. *Journal of clinical sleep medicine, 3*(1): 33-36. https://doi.org/10.5664/jcsm.26742

Eacret, D., Lemchi, C., Caulfield, J. I., Cavigelli, S. A., Veasey, S. C., & Blendy, J. A. (2022). Chronic sleep deprivation blocks voluntary morphine consumption but not conditioned place preference in mice. *Front Neurosci, 16*: 836693. https://doi.org/10.3389/fnins.2022.836693

Evans, B. R., & Leighton, F. A. (2014). A history of one health. *Revue scientifique et technique (International Office of Epizootics), 33*(2): 413-420. https://doi.org/10.20506/rst.33.2.2298

Faradji, H., Cespuglio, R., & Jouvet, M. (1980). Liquid food and tongue activity during sleep and waking in c57br mice. *Physiology & Behavior, 25*(6): 813-817. https://doi.org/10.1016/0031-9384(80)90299-1

Finan, P. H., Goodin, B. R., & Smith, M. T. (2013). The association of sleep and pain: An update and a path forward. *The Journal of Pain, 14*(12): 1539-1552. https://doi.org/10.1016/j.jpain.2013.08.007

Franken, P., Malafosse, A., & Tafti, M. (1998). Genetic variation in eeg activity during sleep in inbred mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *275*(4): R1127-R1137. https://doi.org/10.1152/ajpregu.1998.275.4.R1127

Franken, P., Malafosse, A., & Tafti, M. (1999). Genetics determinants of sleep in regulation in inbred mice. *Sleep*, *22*(2): 155-169. https://doi.org/10.1093/sleep/22.2.155

Freire, C., Sennes, L. U., & Polotsky, V. Y. (2022). Opioids and obstructive sleep apnea. *Journal of clinical sleep medicine*, *18*(2): 647-652. https://doi.org/10.5664/jcsm.9730

Gaulden, A. D., Burson, N., Sadik, N., Ghosh, I., Khan, S. J., Brummelte, S., Kallakuri, S., & Perrine, S. A. (2021). Effects of fentanyl on acute locomotor activity, behavioral sensitization, and contextual reward in female and male rats. *Drug and Alcohol Dependence*, *229*: 109101. https://doi.org/10.1016/j.drugalcdep.2021.109101

Gauthier, E. A., Guzick, S. E., Brummett, C. M., Baghdoyan, H. A., & Lydic, R. (2011). Buprenorphine disrupts sleep and decreases adenosine concentrations in sleep-regulating brain regions of sprague dawley rat. *Anesthesiology*, *115*(4): 743-753. https://doi.org/10.1097/ALN.0b013e31822e9f85

Gaviraghi Mussoi, J., Stanley, M. C., & Cain, K. E. (2022). Importance of sleep for avian vocal communication. *Biology Letters*, *18*(8): 20220223. https://doi.org/10.1098/rsbl.2022.0223

Geller, E. B., Hawk, C., Keinath, S. H., Tallarida, R. J., & Adler, M. W. (1983). Subclasses of opioids based on body temperature change in rats: Acute subcutaneous administration. *Journal of Pharmacology and Experimental Therapeutics*, *225*(2): 391 - 398. http://jpet.aspetjournals.org/content/225/2/391.abstract

Ghose, R., Forati, A. M., & Mantsch, J. R. (2022). Impact of the covid-19 pandemic on opioid overdose deaths: A spatiotemporal analysis. *Journal of urban health*, *99*(2): 316-327.

https://doi.org/10.1007/s11524-022-00610-0

Gilbert, S. S., van den Heuvel, C. J., Ferguson, S. A., & Dawson, D. (2004). Thermoregulation as a sleep signalling system. *Sleep Medicine Reviews, 8*(2): 81-93. https://doi.org/10.1016/S1087-0792(03)00023-6

Goldstein, A., & Sheehan, P. (1969). Tolerance to opioid narcotics. I. Tolerance to the "running fit" caused by levorphanol in the mouse. *Journal of Pharmacology and Experimental Therapeutics, 169*(2): 175-184. https://jpet.aspetjournals.org/content/jpet/169/2/175.full.pdf

Gordon, C. J. (2012). Thermal physiology of laboratory mice: Defining thermoneutrality. *Journal of Thermal Biology, 37*(8): 654-685. https://doi.org/10.1016/j.jtherbio.2012.08.004

Gordon, C. J. (2017). The mouse thermoregulatory system: Its impact on translating biomedical data to humans. *Physiology & Behavior, 179*: 55-66. https://doi.org/10.1016/j.physbeh.2017.05.026

Greening, L., Downing, J., Amiouny, D., Lekang, L., & McBride, S. (2021). The effect of altering routine husbandry factors on sleep duration and memory consolidation in the horse. *Applied Animal Behaviour Science, 236*: 105229. https://doi.org/10.1016/j.applanim.2021.105229

Greenwald, M. K., Moses, T. E. H., & Roehrs, T. A. (2021). At the intersection of sleep deficiency and opioid use: Mechanisms and therapeutic opportunities. *Translational Research, 234*: 58-73. https://doi.org/10.1016/j.trsl.2021.03.006

Greenwald, M. K., & Roehrs, T. A. (2005). Mu-opioid self-administration vs passive administration in heroin abusers produces differential eeg activation. *Neuropsychopharmacology, 30*(1): 212-221. https://doi.org/10.1038/sj.npp.1300596

Gunter, C. (2003). The mighty mouse. *Nature Reviews Genetics, 4*(1): 4-4.

https://doi.org/10.1038/nrg985

Hajiha, M., DuBord, M. A., Liu, H., & Horner, R. L. (2009). Opioid receptor mechanisms at the hypoglossal motor pool and effects on tongue muscle activity in vivo. *The Journal of Physiology*, *587*(11): 2677-2692. https://doi.org/10.1113/jphysiol.2009.171678

Hámor, P. U., Hartmann, M. C., Garcia, A., Liu, D., & Pleil, K. E. (2023). Morphine-context associative memory and locomotor sensitization in mice are modulated by sex and context in a dose-dependent manner. *bioRxiv*: 565492. https://doi.org/10.1101/2023.11.03.565492

Haouzi, P., McCann, M., & Tubbs, N. (2021). Respiratory effects of low and high doses of fentanyl in control and β-arrestin 2-deficient mice. *Journal of Neurophysiology*, *125*(4): 1396-1407. https://doi.org/10.1152/jn.00711.2020

Harding, E. C., Franks, N. P., & Wisden, W. (2019). The temperature dependence of sleep. *Front Neurosci*, *13*: 336. https://doi.org/10.3389/fnins.2019.00336

Harding, E. C., Franks, N. P., & Wisden, W. (2020). Sleep and thermoregulation. *Current Opinion in Physiology*, *15*: 7-13. https://doi.org/10.1016/j.cophys.2019.11.008

Hayat, M. T., Hameed, U., & Zia-Ul-Haq, M. (2023). Opium poppy. In M. Zia-Ul-Haq, A. Abdulkreem Al-Huqail, M. Riaz, & U. Farooq Gohar (Eds.), *Essentials of medicinal and aromatic crops* (pp. 935-964). Springer International Publishing. https://doi.org/10.1007/978-3-031-35403-8_36

Hedegaard, H., Miniño, A. M., Spencer, M. R., & Warner, M. (2021). Drug overdose deaths in the united states, 1999–2020 [Journal Issue]. https://stacks.cdc.gov/view/cdc/112340

Heller, H. C. (1988). Sleep and hypometabolism. *Canadian Journal of Zoology*, *66*(1): 61-69. https://doi.org/10.1139/z88-008

Herrmann, C. S., Strüber, D., Helfrich, R. F., & Engel, A. K. (2016). Eeg oscillations: From

correlation to causality. *International journal of psychophysiology., 103*: 12-21. https://doi.org/10.1016/j.ijpsycho.2015.02.003

Hewes, R. S., & Taghert, P. H. (2001). Neuropeptides and neuropeptide receptors in the drosophila melanogaster genome. *Genome Res, 11*(6): 1126-1142. https://doi.org/10.1101/gr.169901

Hofford, R. S., Wellman, P. J., & Eitan, S. (2012). Morphine alters the locomotor responses to a d2/d3 dopamine receptor agonist differentially in adolescent and adult mice. *Journal of Psychopharmacology, 26*(10): 1355-1365. https://doi.org/10.1177/0269881112443741

Hoskins, B., Burton, C. K., & Ho, I. K. (1986). Differences in morphine-induced antinociception and locomotor activity in mature adult and aged mice. *Pharmacology Biochemistry and Behavior, 25*(3): 599-605. https://doi.org/10.1016/0091-3057(86)90148-6

Huang, R.-C. (2018). The discoveries of molecular mechanisms for the circadian rhythm: The 2017 nobel prize in physiology or medicine. *Biomedical Journal, 41*(1): 5-8. https://doi.org/10.1016/j.bj.2018.02.003

Huber, R., Deboer, T., & Tobler, I. (2000). Topography of eeg dynamics after sleep deprivation in mice. *Journal of Neurophysiology, 84*(4): 1888-1893. https://doi.org/10.1152/jn.2000.84.4.1888

Ikeda, T., Kurz, A., Sessler, D. I., Go, J., Kurz, M., Belani, K., Larson, M., Bjorksten, A. R., Dechert, M., & Christensen, R. (1997). The effect of opioids on thermoregulatory responses in humans and the special antishivering action of meperidine. *Annals of the New York Academy of Sciences, 813*(1): 792-798. https://doi.org/10.1111/j.1749-6632.1997.tb51783.x

Joranson, D. E., Ryan, K. M., Gilson, A. M., & Dahl, J. L. (2000). Trends in medical use and abuse of opioid analgesics. *Jama, 283*(13): 1710-1714. https://doi.org/10.1001/jama.283.13.1710

Judson, B. A., & Goldstein, A. (1978). Genetic control of opiate-induced locomotor activity in mice. *Journal of Pharmacology and Experimental Therapeutics*, *206*(1): 56-60. https://pubmed.ncbi.nlm.nih.gov/207859/

Kalvass, J. C., Olson, E. R., Cassidy, M. P., Selley, D. E., & Pollack, G. M. (2007). Pharmacokinetics and pharmacodynamics of seven opioids in p-glycoprotein-competent mice: Assessment of unbound brain ec_{50,u} and correlation of in vitro, preclinical, and clinical data. *Journal of Pharmacology and Experimental Therapeutics*, *323*(1): 346-355. https://doi.org/10.1124/jpet.107.119560

Karnib, N., Long, B., van Staaden, M., Sprague, J. E., Hall, F. S., Jacobson, D., & Huber, R. (2023). Opiate sensitivity in fruit flies. *Medical Research Archives (Online)*, *11*(4). https://doi.org/10.18103/mra.v11i4.3711

Kest, B., Adler, M., & Hopkins, E. (2000). Sex differences in thermoregulation after acute and chronic morphine administration in mice. *Neuroscience Letters*, *291*(2): 126-128. https://doi.org/10.1016/S0304-3940(00)01393-8

King, L. J. (2012). One health and food safety. Improving Food Safety Through a One Health Approach: Workshop Summary., Washington (DC). https://www.ncbi.nlm.nih.gov/books/NBK114498/

Kitanaka, J., Kitanaka, N., Tomita, K., Hall, F. S., Igarashi, K., Uhl, G. R., & Sato, T. (2023). Glycogen synthase kinase-3 inhibitors block morphine-induced locomotor activation, straub tail, and depression of rearing in mice via a possible central action. *Neurochemical Research*, *48*: 2230 - 2240. https://doi.org/10.1007/s11064-023-03902-2

Koek, W. (2014). Effects of repeated exposure to morphine in adolescent and adult male c57bl/6j mice: Age-dependent differences in locomotor stimulation, sensitization, and body weight

loss. *Psychopharmacology*, *231*(8): 1517-1529. https://doi.org/10.1007/s00213-013-3298-z

Koek, W., France, C. P., & Javors, M. A. (2012). Morphine-induced motor stimulation, motor incoordination, and hypothermia in adolescent and adult mice. *Psychopharmacology*, *219*(4): 1027-1037. https://doi.org/10.1007/s00213-011-2432-z

Kozak, W., Conn, C. A., & Kluger, M. J. (1995). Body temperature, motor activity, and feeding behavior of mice treated with β-chlornaltrexamine. *Physiology & Behavior*, *58*(2): 353-362. https://doi.org/10.1016/0031-9384(95)00067-S

Kurz, A. (2008). Physiology of thermoregulation. *Best Practice & Research Clinical Anaesthesiology*, *22*(4): 627-644. https://doi.org/10.1016/j.bpa.2008.06.004

Kurz, A., & Sessler, D. I. (2003). Opioid-induced bowel dysfunction. *Drugs*, *63*(7): 649-671. https://doi.org/10.2165/00003495-200363070-00003

Kuschinsky, K., & Hornykiewicz, O. (1974). Effects of morphine on striatal dopamine metabolism: Possible mechanism of its opposite effect on locomotor activity in rats and mice. *European Journal of Pharmacology*, *26*(1): 41-50. https://doi.org/10.1016/0014-2999(74)90072-7

Li, Y.-D., Ge, J., Luo, Y.-J., Xu, W., Wang, J., Lazarus, M., Hong, Z.-Y., Qu, W.-M., & Huang, Z.-L. (2020). High cortical delta power correlates with aggravated allodynia by activating anterior cingulate cortex gabaergic neurons in neuropathic pain mice. *Pain*, *161*(2): 288 - 299. https://doi.org/10.1097/j.pain.0000000000001725

Loomis, A. L., Harvey, E. N., & Hobart, G. (1935). Potential rhythms of the cerebral cortex during sleep. *Science*, *81*: 597-598. https://www.jstor.org/stable/1659223

López-Muciño, L. A., García-García, F., Cueto-Escobedo, J., Acosta-Hernández, M., Venebra-Muñoz, A., & Rodríguez-Alba, J. C. (2022). Sleep loss and addiction. *Neuroscience &*

Biobehavioral Reviews, *141*(104832): 1-11. https://doi.org/10.1016/j.neubiorev.2022.104832

Lutz, P.-E., & Kieffer, B. L. (2013). The multiple facets of opioid receptor function: Implications for addiction. *Current Opinion in Neurobiology*, *23*(4): 473-479. https://doi.org/10.1016/j.conb.2013.02.005

Lydic, R., Hillman, D., Jiang, Y., Baghdoyan, H. A., & O'Brien, C. B. (2022). Opiate actions on sleep and breathing. In M. H. Kryger, T. Roth, & C. A. Goldstein (Eds.), *Principles and practice of sleep medicine* (7th edition. ed., Vol. 1, pp. 510-518). Elsevier, New York.

Lydic, R., Keifer, J. C., Baghdoyan, H. A., Craft, R., & Angel, C. (2017). Opiate actions on sleep and breathing. 250-259.e256. https://doi.org/10.1016/B978-0-323-24288-2.00024-6

Matthes, H. W. D., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dollé, P., Tzavara, E., Hanoune, J., Roques, B. P., & Kieffer, B. L. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature*, *383*(6603): 819-823. https://doi.org/10.1038/383819a0

Mehtry, V., Nizamie, S. H., Parvez, N., & Pradhan, N. (2014). Sleep profile in opioid dependence: A polysomnographic case–control study. *Journal of Clinical Neurophysiology*, *31*(6): 517 - 522. https://doi.org/10.1097/WNP.0000000000000117

Miglécz, J. I. S. E., & Rónai, A. Z. (1980). Biphasic effects of a potent enkephalin analogue (d-met2,pro5)-enkephalinamide and morphine on locomotor activity in mice. *Psychopharmacology*, *71*(3): 299-301. https://doi.org/10.1007/BF00433065

Mitler, M. M., Lund, R., Sokolove, P. G., Pittendrigh, C. S., & Dement, W. C. (1977). Sleep and activity rhythms in mice: A description of circadian patterns and unexpeted disruptions of

sleep. *Brain Res*, *131*: 129-145. https://doi.org/10.1016/0006-8993(77)90033-6

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr Opin Plant Biol*, *11*(3): 266-277. https://doi.org/10.1016/j.pbi.2008.03.006

Montandon, G., & Horner, R. L. (2019). Electrocortical changes associating sedation and respiratory depression by the opioid analgesic fentanyl. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-50613-2

Moore, P., & Dimsdale, J. E. (2002). Opioids, sleep, and cancer-related fatigue. *Medical Hypotheses*, *58*(1): 77-82. https://doi.org/10.1054/mehy.2001.1461

Morimoto, S., Suemori, K., Moriwaki, J., Taura, F., Tanaka, H., Aso, M., Tanaka, M., Suemune, H., Shimohigashi, Y., & Shoyama, Y. (2001). Morphine metabolism in the opium poppy and its possible physiological function: Biochemical characterization of the morphine metabolite, bismorphine*. *Journal of Biological Chemistry*, *276*(41): 38179-38184. https://doi.org/10.1074/jbc.M107105200

National Center for Drug Abuse Statistics, N. (2023, n.d.). *Drug overdose death rates*. https://drugabusestatistics.org/drug-overdose-deaths/

Nayak, C. S., & Anilkumar, A. C. (2024). *Eeg normal waveforms* (T. I. (FL), Ed.). StatPearls Publishing Copyright © 2024, StatPearls Publishing LLC. https://www.ncbi.nlm.nih.gov/books/NBK539805/

Nuwer, M. R. (1988). Quantitative eeg: I. Techniques and problems of frequency analysis and topographic mapping. *Journal of Clinical Neurophysiology*, *5*(1): 1-44. https://doi.org/10.1097/00004691-198801000-00001

O'Brien, C. B., Locklear, C. E., Glovak, Z. T., Zebadúa Unzaga, D., Baghdoyan, H. A., & Lydic, R. (2021). Opioids cause dissociated states of consciousness in c57bl/6j mice. *Journal of*

Neurophysiology, *126*(4): 1265-1275. https://doi.org/10.1152/jn.00266.2021

Okamoto-Mizuno, K., & Mizuno, K. (2012). Effects of thermal environment on sleep and circadian rhythm. *J Physiol Anthropol*, *31*(1): 14. https://doi.org/10.1186/1880-6805-31-14

Oliverio, A., & Castellano, C. (1974). Genotype-dependent sensitivity and tolerance to morphine and heroin: Dissociation between opiate-induced running and analgesia in the mouse. *Psychopharmacologia*, *39*(1): 13-22. https://doi.org/10.1007/BF00421454

Oliverio, A., Castellano, C., & Puglisi-Allegra, S. (1982). Opiate analgesia: Evidence for circadian rhythms in mice. *Brain Research*, *249*(2): 265-270. https://doi.org/10.1016/0006-8993(82)90060-9

Osman, I., Nadir, Baghdoyan, H. A., & Lydic, R. (2005). Morphine inhibits acetylcholine release in rat prefrontal cortex when delivered systemically or by microdialysis to basal forebrain. *Anesthesiology*, *103*(4): 779-787. https://doi.org/10.1097/00000542-200510000-00016

Panagiotou, M., Vyazovskiy, V. V., Meijer, J. H., & Deboer, T. (2017). Differences in electroencephalographic non-rapid-eye movement sleep slow-wave characteristics between young and old mice. *Scientific Reports*, *7*(1). https://doi.org/10.1038/srep43656

Patti, C. L., Frussa-Filho, R., Silva, R. H., Carvalho, R. C., Kameda, S. R., Takatsu-Coleman, A. L., Cunha, J. L. S., & Abílio, V. C. (2005). Behavioral characterization of morphine effects on motor activity in mice. *Pharmacology Biochemistry and Behavior*, *81*(4): 923-927. https://doi.org/10.1016/j.pbb.2005.07.004

Paul, A. K., Smith, C. M., Rahmatullah, M., Nissapatorn, V., Wilairatana, P., Spetea, M., Gueven, N., & Dietis, N. (2021). Opioid analgesia and opioid-induced adverse effects: A review. *Pharmaceuticals*, *14*(11): 1091. https://doi.org/10.3390/ph14111091

Pert, A., Dewald, L. A., Liao, H., & Sivit, C. (1979). Effects of opiates and opioid peptides on

motor behaviors: Sites and mechanisms of action. In E. Usdin, W. E. Bunney, & N. S. Kline (Eds.), *Endorphins in mental health research* (pp. 45-61). Palgrave Macmillan UK. https://doi.org/10.1007/978-1-349-04015-5_4

Pickworth, W. B., & Sharpe, L. G. (1979). Eeg-behavioral dissociation after morphine and cyclazocine-like drugs in the dog: Further evidence for two opiate receptors. *Neuropharmacology*, *18*(7): 617-622. https://doi.org/10.1016/0028-3908(79)90114-X

Prerau, M. J., Brown, R. E., Bianchi, M. T., Ellenbogen, J. M., & Purdon, P. L. (2017). Sleep neurophysiological dynamics through the lens of multitaper spectral analysis. *Physiology (Bethesda, Md.)*, *32*(1): 60-92. https://doi.org/10.1152/physiol.00062.2015

Rabinowitz, P. M., Kock, R., Kachani, M., Kunkel, R., Thomas, J., Gilbert, J., Wallace, R., Blackmore, C., Wong, D., Karesh, W., Natterson, B., Dugas, R., & Rubin, C. (2013). Toward proof of concept of a one health approach to disease prediction and control. *Emerg Infect Dis*, *19*(12). https://doi.org/10.3201/eid1912.130265

Raffaeli, W., Marconi, G., Fanelli, G., Taddei, S., Borghi, G. B., & Casati, A. (2006). Opioid-related side-effects after intrathecal morphine: A prospective, randomized, double-blind dose–response study. *European Journal of Anaesthesiology | EJA*, *23*(7): 605 - 610. https://doi.org/10.1017/S026502150600038X

Rawls, S. M., & Benamar, K. (2011). Effects of opioids, cannabinoids, and vanilloids on body temperature. *FBS*, *3*(3): 822-845. https://doi.org/10.2741/190

Reid-Varley, W. B., Ponce Martinez, C., & Khurshid, K. A. (2020). Sleep disorders and disrupted sleep in addiction, withdrawal and abstinence with focus on alcohol and opioids. *J Neurol Sci*, *411*: 116713. https://doi.org/10.1016/j.jns.2020.116713

Richardson, G. S., Moore-Ede, M. C., Czeisler, C. A., & Dement, W. C. (1985). Circadian rhythms

of sleep and wakefulness in mice: Analysis using long-term automated recording of sleep. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 248*(3): R320-R330. https://doi.org/10.1152/ajpregu.1985.248.3.R320

Rikard, S., Strahan, A., Schmit, K., & Guy, G. J. (2023). Chronic pain among adults — united states, 2019–2021. *MMWR. Morbidity and mortality weekly report, 72*: 379 - 385. https://doi.org/10.15585/mmwr.mm7215a1

Roehrs, T., Sibai, M., & Roth, T. (2021). Sleep and alertness disturbance and substance use disorders: A bi-directional relation. *Pharmacology Biochemistry and Behavior, 203*(173153): 1-8. https://doi.org/10.1016/j.pbb.2021.173153

Romanovsky, A. A. (2018). *The thermoregulation system and how it works* (Vol. 156). Elsevier. https://doi.org/10.1016/B978-0-444-63912-7.00001-1

Rosow, C. E., Miller, J. M., Pelikan, E. W., & Cochin, J. (1980). Opiates and thermoregulation in mice. I. Agonists. *Journal of Pharmacology and Experimental Therapeutics, 213*(2): 273-283. https://jpet.aspetjournals.org/content/213/2/273

Rosow, C. E., Miller, J. M., Poulsen-Burke, J., & Cochin, J. (1982). Opiates and thermoregulation in mice. Iv. Tolerance and cross-tolerance. *Journal of Pharmacology and Experimental Therapeutics, 223*(3): 702. http://jpet.aspetjournals.org/content/223/3/702.abstract

Rothhaas, R., & Chung, S. (2021). Role of the preoptic area in sleep and thermoregulation [Review]. *Frontiers in Neuroscience, 15*. https://doi.org/10.3389/fnins.2021.664781

Rummans, T. A., Burton, M. C., & Dawson, N. L. (2018). How good intentions contributed to bad outcomes: The opioid crisis. *Mayo Clinic Proceedings, 93*(3): 344-350. https://doi.org/10.1016/j.mayocp.2017.12.020

Sadowski, B., Sas, R., Swiderski, T., & Trzasko, M. (1996). Differentiation of opioid and

nonopioid pain inhibitory mechanisms, and of thermoregulation in mice selectively bred for high and low swim stress-induced analgesia. *Analgesia, 2*(4): 211-218. https://www.ingentaconnect.com/contentone/cog/ana/1996/00000002/00000004/art00005

Santos, E. J., Banks, M. L., & Negus, S. S. (2022). Role of efficacy as a determinant of locomotor activation by mu opioid receptor ligands in female and male mice. *Journal of Pharmacology and Experimental Therapeutics, 382*(1): 44-53. https://doi.org/10.1124/jpet.121.001045

Sateia, M. J., & Thorpy, M. J. (2017). Classification of sleep disorders. 618-626.e614. https://doi.org/10.1016/B978-0-323-24288-2.00061-1

Savić Vujović, K. R., Vučković, S., Srebro, D., Ivanović, M., Došen-Mićović, L., Vučetić, Č., Džoljić, E., & Prostran, M. (2013). A comparison of the antinociceptive and temperature responses to morphine and fentanyl derivatives in rats. *Archives of Pharmacal Research, 36*(4): 501-508. https://doi.org/10.1007/s12272-013-0072-z

Scheffzük, C., Kukushka, V. I., Vyssotski, A. L., Draguhn, A., Tort, A. B. L., & Brankačk, J. (2011). Selective coupling between theta phase and neocortical fast gamma oscillations during rem-sleep in mice. *PLoS ONE, 6*(12): e28489. https://doi.org/10.1371/journal.pone.0028489

Serafini, R. A., Pryce, K. D., & Zachariou, V. (2020). The mesolimbic dopamine system in chronic pain and associated affective comorbidities. *Biological Psychiatry, 87*(1): 64-73. https://doi.org/10.1016/j.biopsych.2019.10.018

Shepard Jr, J. W., Buysse, D. J., Chesson Jr, A. L., Dement, W. C., Goldberg, R., Guilleminault, C., Harris, C. D., Iber, C., Mignot, E., Mitler, M. M., Moore, K. E., Phillips, B. A., Quan, S. F., Rosenberg, R. S., Roth, T., Schmidt, H. S., Silber, M. H., Walsh, J. K., & White, D.

P. (2005). History of the development of sleep medicine in the united states. *Journal of clinical sleep medicine, 1*(1): 61-82. https://doi.org/10.5664/jcsm.26298

Siegel, J. M. (2008). Do all animals sleep? *Trends in Neurosciences, 31*(4): 208-213. https://doi.org/10.1016/j.tins.2008.02.001

Siegfried, B., Alleva, E., & Oliverio, A. (1980). An analysis of neurophysiological and behavioral arousal in the mouse. *Physiology & Behavior, 25*(3): 421-424. https://doi.org/10.1016/0031-9384(80)90283-8

Spencer, G. (2012, May 23, 2012). *Background on mouse as a model organism*. National Human Genome Research Institute. https://www.genome.gov/10005834/background-on-mouse-as-a-model-organism

Srivastava, A. B., & Gold, M. S. (2018). Beyond supply: How we must tackle the opioid epidemic. *Mayo Clinic Proceedings, 93*(3): 269-272. https://doi.org/10.1016/j.mayocp.2018.01.018

Stepanski, E. J. (2002). The effect of sleep fragmentation on daytime function. *Sleep, 25*(3): 268-276.

Torralva, R., & Janowsky, A. (2019). Noradrenergic mechanisms in fentanyl-mediated rapid death explain failure of naloxone in the opioid crisis. *Journal of Pharmacology and Experimental Therapeutics, 371*(2): 453. https://doi.org/10.1124/jpet.119.258566

Toth, L. A. (2023). Opinion: Laboratory animals, animal models, or just animals? *J Am Assoc Lab Anim Sci, 62*(3). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10230533/

Tripathi, R., Rao, R., Dhawan, A., Jain, R., & Sinha, S. (2020). Opioids and sleep - a review of literature. *Sleep Medicine, 67*(3): 269-275. https://doi.org/10.1016/j.sleep.2019.06.012

Vadhel, A., Bashir, S., Mir, A. H., Girdhar, M., Kumar, D., Kumar, A., Mohan, A., Malik, T., & Mohan, A. (2023). Opium alkaloids, biosynthesis, pharmacology and association with

cancer occurrence. *Open Biol*, *13*(5): 220355. https://doi.org/10.1098/rsob.220355

Valatx, J. L., & Bugat, R. (1974). [genetic factors as determinants of the waking-sleep cycle in the mouse (author's transl)]. *Brain Research*, *69*(2): 315-330. https://doi.org/10.1016/0006-8993(74)90009-2

Valatx, J. L., Bugat, R., & Jouvet, M. (1972). Genetic studies of sleep in mice. *Nature*, *238*(5361): 226-227. https://doi.org/10.1038/238226a0

Varshneya, N. B., Walentiny, D. M., Moisa, L. T., Walker, T. D., Akinfiresoye, L. R., & Beardsley, P. M. (2019). Opioid-like antinociceptive and locomotor effects of emerging fentanyl-related substances. *Neuropharmacology*, *151*: 171-179. https://doi.org/10.1016/j.neuropharm.2019.03.023

Varshneya, N. B., Walentiny, D. M., Moisa, L. T., Walker, T. D., Akinfiresoye, L. R., & Beardsley, P. M. (2021). Fentanyl-related substances elicit antinociception and hyperlocomotion in mice via opioid receptors. *Pharmacology Biochemistry and Behavior*, *208*: 173242. https://doi.org/10.1016/j.pbb.2021.173242

Volkow, N. D., & McLellan, A. T. (2016). Opioid abuse in chronic pain — misconceptions and mitigation strategies. *New England Journal of Medicine*, *374*(13): 1253-1263. https://doi.org/10.1056/NEJMra1507771

Wanke, T., Lahrmann, H., Formanek, D., Zwick, B., Merkle, M., & Zwick, H. (1993). The effect of opioids on inspiratory muscle fatigue during inspiratory resistive loading. *Clinical physiology (Oxford)*, *13*(4): 349-360. https://doi.org/10.1111/j.1475-097X.1993.tb00335.x

Weir, K. (2022). Diagnosing and treating sleep disorders. *Monitor on Psychology*, *53*(5). https://www.apa.org/monitor/2022/07/ce-sleep-disorders

Welsh, D. K., Engle, E. M. R. A., Richardson, G. S., & Dement, W. C. (1986). Precision of

circadian wake and activity onset timing in the mouse. *Journal of Comparative Physiology A, 158*(6): 827-834. https://doi.org/10.1007/BF01324824

Welsh, D. K., Richardson, G. S., & Dement, W. C. (1985). A circadian rhythm of hippocampal theta activity in the mouse. *Physiology & Behavior*, *35*(4): 533-538. https://doi.org/10.1016/0031-9384(85)90136-2

Wesensten, N. J., Balkin, T. J., & Belenky, G. (1999). Does sleep fragmentation impact recuperation?A review and reanalysis. *Journal of sleep research, 8*(4): 237-245. https://doi.org/10.1046/j.1365-2869.1999.00161.x

Wheeler, A. G. J. (2019). Bugs and poppies: Heteroptera associated with developing and mature capsules (fruits) of papaver somniferum l. (papaveraceae). *Proceedings of the Entomological Society of Washington*, *121*(3): 511-524. https://doi.org/10.4289/0013-8797.121.3.511

Wink, M. (2018). Plant secondary metabolites modulate insect behavior-steps toward addiction? *Frontiers in physiology, 9*: 364-364. https://doi.org/10.3389/fphys.2018.00364

Yeshi, K., Crayn, D., Ritmejerytė, E., & Wangchuk, P. (2022). Plant secondary metabolites produced in response to abiotic stresses has potential application in pharmaceutical product development. *Molecules*, *27*(1).

Zebadúa Unzaga, D., Bustamante, C., Thibert, M., & Baghdoyan, H. A. (2021). Fentanyl and morphine cause dose-dependent sleep disruption in c57bl/6j (b6) mice. *The FASEB Journal, 35*. https://doi.org/10.1096/fasebj.2021.35.S1.01706

Zebadúa Unzaga, D., Xiaojuan, Z., Joshua, M. P., Michael, A. O., Ralph, L., & Helen, A. B. (2023). Mediation analysis reveals that opioid-induced sleep disruption is partially mediated by increased motor activity in c57bl/6j (b6) mice. *Journal of Pharmacology and*

Experimental Therapeutics, *385*(S3): 542. https://doi.org/10.1124/jpet.122.192550

Zebadúa Unzaga, D., Zhu, X., Price, J., Lydic, R., & Baghdoyan, H. A. (2022). Fentanyl-induced sleep disruption in c57bl/6j (b6) mice is not secondary to increased motor activity or hypothermia [Abstract]. *The FASEB Journal*, *36*(S1). https://doi.org/10.1096/fasebj.2022.36.S1.R2168

Zhang, J.-J., & Kong, Q. (2017). Locomotor activity: A distinctive index in morphine self-administration in rats [Report]. *PLoS ONE*, *12*: e0174272. http://dx.doi.org.utk.idm.oclc.org/10.1371/journal.pone.0174272

Appendix

Figure 2.1. Implanted mouse in home cage placed over a recording pad.

Chapter 2 : Opioids cause dissociated states of consciousness in C57BL/6J mice

This chapter has been adapted for dissertation formatting. A version of this chapter was originally published by:

O'Brien CB, Locklear CE, Glovak ZT, **Zebadúa Unzaga D**, Baghdoyan HA, Lydic, R. Opioids cause dissociated states of consciousness in C57BL/6J mice.

J Neurophysiol 126:1265-1275, 2021 https://doi.org/10.1152/jn.00266.2021

My contributions to the publication presented and adapted in the following chapter include data collection, analysis, and interpretation. Additionally, I contributed to the writing of manuscript and figure preparation.

Abstract

The electroencephalogram (EEG) provides an objective, neural correlate of consciousness. Opioid receptors modulate mammalian neuronal excitability, and this fact was used to characterize how opioids administered to mice alter EEG power and states of consciousness. The present study tested the hypothesis that antinociceptive doses of fentanyl, morphine, or buprenorphine differentially alter the EEG and states of sleep and wakefulness in adult, male C57BL/6J mice. Mice were anesthetized and implanted with telemeters that enabled wireless recordings of cortical EEG and electromyogram (EMG). After surgical recovery, EEG and EMG were used to objectively score states of consciousness as wakefulness, rapid eye movement (REM) sleep, or non-REM (NREM) sleep. Measures of EEG power (dB) were quantified as delta (0.5 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 13 Hz), sigma (12 to 15 Hz), beta (13 to 30 Hz), and gamma (30 to 60 Hz). Compared to saline (control), fentanyl and morphine decreased NREM sleep, morphine eliminated REM sleep, and buprenorphine eliminated NREM sleep and REM sleep. Opioids significantly and differentially disrupted the temporal organization of sleep/wake states, altered specific EEG frequency bands, and caused dissociated states of

consciousness. The results are discussed relative to the fact that opioids, pain, and sleep modulate interacting states of consciousness.

Introduction

An unresolved challenge for consciousness studies is understanding how subjective phenomenology relates to empiric neurophysiology. In contrast, physiology, pharmacology, and pathology that significantly change brain function are universally understood to alter states of consciousness. An association between states of consciousness and the electroencephalogram (EEG) was demonstrated in 1935 by the first human EEG recording during sleep (Loomis et al., 1935). Two decades later the EEG was used to identify heterogenous sleep states (Aserinsky & Kleitman, 1953) that were subsequently related to different subjective measures of consciousness (Dement & Kleitman, 1957). The discovery of remarkable homology between human and cat EEG (Dement, 1958) supported the interpretation that EEG-based studies of non-human animals can provide novel insights into the neurophysiology underlying states of consciousness. Today, that inference seems obvious relative to evidence that approximately 90 percent of the human genome has similar genes shared by cat (Pontius et al., 2007) and mouse (Monaco et al., 2015). Long before derivation of the mouse genome (Mouse Genome Sequencing Consortium, 2002), prescient investigators recognized the potential for studies of mice to provide insights into the genetic and pharmacogenetic modulation of EEG activity during sleep and wakefulness (Franken et al., 1998; Mitler et al., 1977; Richardson et al., 1985; Valatx et al., 1972; Wimer et al., 1969). Contemporary efforts to better understand opioid use disorder and opioid-induced respiratory depression are being advanced by studies of wild type, congenic, and transgenic mice (Angel et al., 2018; Charbogne et al., 2014; Lydic & Baghdoyan, 2021; Ramirez et al., 2021; Varga et al., 2020).

Opioids alter states of consciousness and, by disrupting sleep, contribute to cognitive dysfunction (Lutz & Kieffer, 2013) and the perpetuation of addiction (Valentino & Volkow, 2020). No previous studies have quantified how different opioids alter EEG defined states of consciousness in wild type mice. To address this gap in knowledge, the present study tested the hypothesis that antinociceptive doses of fentanyl, morphine, and buprenorphine differentially alter the EEG, sleep, and wakefulness in C57BL/6J mice.

Methods

Animals and Animal Care

All procedures in this study that involved animals were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee and adhered to the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 8th ed., Washington, DC, 2011). Adult, male, C57BL/6J (B6; Stock# 000664; n = 10) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mean ± SD age of the mice was 36.4 ± 10.9 weeks and mean ± SD body weight was 36.2 ± 4.5 g. Upon arrival, mice were housed in standard cages (19 x 29 x 13 cm) with no more than 5 littermates per cage and were given at least one week for adaptation to the laboratory environment. Enrichment included Igloos and a rotation between nestlets and cones.

Cage size and enrichment permitted digging, nest building, and mouse-specific sleep postures. The housing facility provided a temperature (average 23°C) and humidity (average 45%) controlled environment with 10 to 15 complete air changes per h and a 12-h light:12-h dark cycle. Wi-Fi connections between mouse room sensors and investigator cell phones provided 24-h monitoring of environmental conditions. Mice had ad libitum access to water and food (Teklad 8640 rodent chow, Envigo, Madison, WI). All mice were checked daily by the

investigators and regularly by University of Tennessee Office of Laboratory Animal Care staff. Harlan Soft Cob Enrichment Bedding was replaced weekly. After surgical implantation of recording electrodes, mice were housed individually to prevent them from removing sutures and/or wound clips from cage mates.

Surgical Implantation of Electrodes and Telemeters

Using surgical procedures described previously (O'Brien et al., 2019; Zhang et al., 2020), mice were deeply anesthetized with 2% isoflurane (Henry Schein, Melville, NY) delivered in 100% oxygen at a flow rate of 1 L/min. Loss of righting reflex was used as a surrogate measure to confirm loss of consciousness (Vanini et al., 2014). When unconsciousness was achieved, mice were removed from the induction chamber and isoflurane delivery was continued via a nose cone. Ketoprofen (5 mg/kg) was administered subcutaneously, and mice were shaved and scrubbed for surgery. Mice were then stabilized in a stereotaxic frame (David Kopf Instruments, Tujunga CA; model 962) fitted with a mouse adaptor (model 921) and a mouse anesthesia mask (model 907). Delivered isoflurane concentration was maintained at 1.5% by spectrophotometry (Cardiocap/5; Datex-Ohmeda Inc., Madison WI). Core body temperature was maintained at 36-37°C by placing mice on a heating pad filled with circulating hot water (Gaymar TP400 T/Pump heath therapy system). Respiratory rate, core body temperature, and delivered isoflurane concentration were recorded every 10 min. Paw withdrawal in response to pinch of a hind paw was used to confirm antinociception.

A dorsal skin incision was made from the top of the skull to the midpoint between the scapulae, and a subcutaneous pocket was made on the dorsal flank. Bipolar electrodes for recording the EEG were placed under the skull at stereotaxic coordinates 3.0 mm posterior to bregma and 3.0 mm lateral to the midline, and 1 mm anterior to bregma and 1 mm lateral to the midline (Paxinos

& Franklin, 2019). A pair of electrodes for recording the electromyogram (EMG) were implanted in a dorsal neck muscle. EEG and EMG electrodes were connected to a telemeter (HD-X02, Data Sciences International, DSI, New Brighton, MN) that was implanted subcutaneously in the dorsal flank. Each mouse received a subcutaneous radio-frequency identification chip (ID-100B 1.4, MICROCHIPID, Lake Zurich, IL) for unique alphanumeric identification. The incision was closed using wound clips and sutures (Prolene 6-0; Henry Schein) and isoflurane was discontinued. Mice were placed in a heated cage and monitored continuously. When they ambulated normally, mice were returned to their home cages and had a minimum of seven days to recover from surgery. The recovery period included time to adapt to being individually housed.

Data Acquisition and Signal Processing

Before every recording session, the home cage of each mouse was placed on a pad containing an antenna that received EEG and EMG signals from the telemeter. The receiver pads were connected to a DSI Matrix 2.0 (MX2) allowing for communication between the telemetry implants and the data acquisition computer, which was outside of the recording room. The EEG and EMG signals were digitized using Ponemah software (v6.33, DSI) and acquired at a sampling frequency of 500 Hz. Raw recorded data were imported into Neuroscore (v3.3.1, DSI) for manual scoring of states of consciousness. For scoring, bandpass filters were applied to the EEG (0.3 to 30 Hz) and EMG (10 to 100 Hz) signals. A notch filter (60 Hz) was applied to both signals to reduce electrical noise.

Assessing States of Consciousness

EEG and EMG signals were used to objectively score states of wakefulness, rapid eye movement (REM) sleep, and non-REM (NREM) sleep for every 10-s epoch of recording. Scoring criteria

for each state of consciousness have been described (Flint et al., 2010). Briefly, wakefulness was characterized by low amplitude EEG and the presence of maximal EMG signal. NREM sleep was identified by increased EEG delta (0.5 to 4 Hz) activity and reduced muscle tone. Epochs of REM sleep showed increased theta (4 to 8 Hz) EEG frequency content and muscle atonia. **Figure 2.7** shows EEG and EMG signals that illustrate visualizable differences among wakefulness, NREM sleep, and REM sleep (https://doi.org/10.6084/m9.figshare.15094251). For an epoch to be scored as a certain state of consciousness, greater than half of the 10-s epoch must have comprised that state. Each recording was scored by two individuals, and one scorer was blinded to the treatment condition. The state scoring between two experimenters was required to achieve ≥90% concordance. Recording duration was 4 h or 24 h.

Experimental Procedure and Design

All experiments began approximately 1.5 h after light onset. States of sleep and wakefulness were recorded immediately following intraperitoneal injection (0.3 mL) of saline (vehicle control) or a mouse antinociceptive dose of fentanyl (0.4 mg/kg) (Fechtner et al., 2015), morphine (10 mg/kg) (Gades et al., 2000), or buprenorphine (0.3 mg/kg) (Lutfy et al., 2003). The 4-h effects of opioids were studied in 10 mice, and 24-h effects were quantified using 6 of the 10 mice. Sample sizes were chosen based on analysis of preliminary data (Locklear et al., 2019; O'Brien et al., 2021; Zebadúa Unzaga et al., 2021). A within-subjects design was used such that each mouse received at least one injection of saline and each opioid. The order of opioid administration was randomized, and seven days without drug treatment were given between opioid injections into the same mouse. At the end of the study, mice were deeply anesthetized with isoflurane then euthanized by decapitation.

Spectral Analysis of EEG Data

Cortical EEG signals are comprised of differing oscillations, and drugs can cause changes in EEG frequency, amplitude, and power. Opioid-induced changes in EEG were analyzed using multitaper spectral estimation and by dividing the signals into previously established power bands (Prerau et al., 2017). Multitaper spectral estimation is a branch of Fourier analysis and is useful for examining time domain signals.

Unfiltered EEG data were exported as European Data Format files from the Neuroscore program. Data were analyzed in MATLAB (R2020b, MathWorks) using customs scripts (O'Brien et al., 2019) and the Chronux toolbox (http://chronux.org). Spectral analysis involved examining EEG content ranging from 0 to 60 Hz, using a 3-s window that advanced in 0.1-s steps, a time-bandwidth product of 2, and 3 discrete prolate spheroidal sequence tapers. EEG power (dB) was averaged and quantified in six frequency bands: delta (0.5 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 13 Hz), sigma (12 to 15 Hz), beta (13 to 30 Hz), and gamma (30 to 60 Hz) (Prerau et al., 2017).

Statistical Analyses

Prism (v8.2.1, GraphPad, San Diego, CA) was used to perform all statistics. Dependent variables evaluated included the percent of time spent in each state of consciousness, number of episodes for each state, duration of the state episodes, and latency to onset of NREM sleep and REM sleep. Sleep latencies were calculated from the time of injection. The effects of opioids on the foregoing measures were analyzed using repeated measures, one-way or two-way ANOVA. Post-hoc comparisons were performed using Tukey's multiple comparisons test. The same analyses were applied to measures of EEG spectral power. Effect sizes were determined using the Eta squared statistic. For all comparisons, a probability (P) value < 0.05 was considered statistically significant. Data are expressed as median with interquartile range (IQR). Radar

plots were constructed using MATLAB (https://github.com/NewGuy012/spider_plot).

Results

Opioids Disrupted the Temporal Organization of Sleep and Wakefulness

Figure 2.1 shows data from one representative mouse to illustrate opioid-induced changes in sleep/wake architecture (left column) and EEG power (right column) relative to control (Fig. 2.1A). **Figure 2.8** shows EEG and EMG signals from the **Fig. 2.1** mouse during wakefulness 10 min after administration, on different days, of saline and each of the opioids. For 4 h after injection, fentanyl (**Fig. 2.1B**) and morphine (**Fig. 2.1C**), decreased NREM sleep and eliminated REM sleep. Buprenorphine (**Fig. 2.1D**) eliminated NREM sleep and REM sleep. The spectrograms illustrate that each opioid produced unique changes in EEG power.

Figure 2.2 summarizes the effects of opioids on the percent of time mice spent in each state, number of sleep/wake episodes, and episode duration during the 4-h recording period. Each opioid significantly increased wakefulness relative to saline (**Fig. 2.2A**). NREM sleep (**Fig. 2.2B**) was significantly reduced by fentanyl and morphine, and eliminated by buprenorphine. REM sleep (**Fig. 2.2C**) was decreased by fentanyl and eliminated by morphine and buprenorphine. Opioids decreased the number of episodes of wakefulness (**Fig. 2.2D**), NREM sleep (**Fig. 2.2E**), and REM sleep (**Fig. 2.2F**). Opioids increased the duration of wakefulness (**Fig. 2.2G**) and decreased the duration of NREM sleep (**Fig. 2.2H**) and REM sleep (**Fig. 2.2I**). Descriptive statistics for all data in **Fig. 2.2** are provided in Supplemental **Table S2.1.** F-values, numerator (n) and denominator (d) degrees of freedom (DF), P-values, and effect sizes (Eta squared) for the **Fig. 2.2** results are reported in Supplemental **Table S2.2.**

Opioids Disrupted States of Consciousness for 24 Hours

Figure 2.3 histograms plot the 24-h distribution of wakefulness, NREM sleep, and REM sleep

from one representative mouse. The histograms illustrate the temporal organization for these states of consciousness after injection (hour 0) of saline (**Fig. 2.3A**), fentanyl (**Fig. 2.3B**), morphine (**Fig. 2.3C**), and buprenorphine (**Fig. 2.3D**). The gray screen indicates the dark phase of the light:dark cycle.

Figure 2.4 illustrates the effects of opioids on states of consciousness across the 24-h light:dark cycle. The three rows plot different dependent measures for wakefulness (**Fig. 2.4 A, D, G**), NREM sleep (**Fig. 2.4 B, E, H**), and REM sleep (**Fig. 2.4 C, F, I**). Columns show measures acquired during the light phase (no gray screen) and dark phase (gray screen). Descriptive statistics for the **Fig. 2.4** data are available in **Supplemental Table S2.3**. F-values, numerator (n) and denominator (d) degrees of freedom (DF), P-values, and measures of effect size (Eta squared) for the **Fig. 2.4** data are provided in **Supplemental Table S2.4.**

Figure 2.4A shows that, relative to saline, each of the three opioids increased time spent awake during the light period when mice normally sleep, and decreased wakefulness during the dark phase when mice are normally most active. Corresponding opioid-induced decreases in NREM sleep (**Fig. 2.4B**) and REM sleep (**Fig. 2.4C**) occurred during the light phase, followed by rebound increases in both stages of sleep during the dark phase.

The effects of opioids on the number of episodes of wakefulness, NREM sleep, and REM sleep during one light:dark cycle are represented in the middle row of **Fig. 2.4.** The number of waking episodes was decreased by all opioids during the light phase (**Fig. 2.4D**) and was not different from control during the dark phase (**Fig. 2.4D**, gray screen). Similarly, the number of NREM sleep episodes was decreased during the light phase and not significantly altered during the dark period (**Fig. 2.4E**). The number of REM sleep episodes during the light phase was significantly decreased by all opioids (**Fig. 2.4F**). The number of REM sleep episodes significantly increased

during the dark period (**Fig. 2.4F**).

Fig. 2.4G – I plots episode duration and shows that buprenorphine significantly increased the duration of wakefulness only during the light phase. NREM sleep episode duration was significantly increased by fentanyl and morphine and decreased by buprenorphine during the light phase (**Fig. 2.4H**). Relative to saline, REM sleep episode duration was increased by fentanyl and morphine and decreased by buprenorphine during the light phase (**Fig. 2.4I**). There was no effect of opioids on REM sleep duration during the dark phase (**Fig. 2.4I**).

Time Course of Opioid-Induced Sleep Disruption

The **Figure 2.5** radar plots illustrate the time course of opioid effects on percent of time spent in wakefulness (**Fig. 2.5A**), NREM sleep (**Fig. 2.5B**), and REM sleep (**Fig. 2.5C**). Numbers around the circumference of each radar plot indicate time (h) after injection. Mean values are shown for percent state after injection of saline (black), fentanyl (red), morphine (green), and buprenorphine (blue). Injection of saline and opioids occurred during the light phase at time 0. EEG recordings began immediately after injection. Reading in a clockwise direction, the light phase ended 10.5 h after injection (radial point 10.5). The thick black line around the left half of each plot indicates the dark phase. The dark phase ended 12 h later at 22.5 h after injection. The gray fill indicates the control values.

The functions plotted in colors show that opioids caused a prolonged increase in wakefulness during the light phase (**Fig. 2.5A**) when mice typically sleep. Morphine and fentanyl increased wakefulness for 5 to 6 h, respectively, whereas buprenorphine increased wakefulness for 11 h. The concomitant opioid-induced decrease in NREM sleep (**Fig. 2.5B**) can be seen by comparing the red, green, and blue lines with the gray filled area (saline). Morphine and fentanyl decreased NREM sleep for 5 to 6 h, respectively, whereas buprenorphine decreased NREM sleep for 10 h.

The rebound increase in NREM sleep and REM sleep plotted as a function of opioid (**Fig. 2.4B - C**) is shown in **Fig. 2.5B - C** as a function of time after opioid administration. The time course plots show that opioids caused NREM sleep (**Fig. 2.5B**) and REM sleep (**Fig. 2.5C**) to be shifted to the dark phase, a time when mice are normally most active.

Opioids also increased the latency from time of injection to onset of both NREM sleep (**Fig. 2.5D**) and REM sleep (**Fig. 2.5E**). Differential effects among opioids were due to buprenorphine, which caused a significantly greater increase in latency than fentanyl or morphine. Descriptive statistics for **Figs. 2.5A – C** are in **Supplemental Table S2.5.1.** Descriptive statistics for **Figs. 2.5D and E** are reported in **Supplemental Table S2.5.2**. F-values, numerator (n) and denominator (d) degrees of freedom (DF), P-values, and effect size (Eta squared) for the **Fig. 2.5** results are in **Supplemental Table S2.6.**

EEG Power was Differentially Altered by Fentanyl, Morphine, and Buprenorphine

EEG power was analyzed during the first 30 min after each treatment condition when all mice were awake. **Figures 2.6A – D** plot spectral power across frequencies ranging from 0 to 60 Hz as median (solid line) and interquartile range (shaded area). EEG spectral power was most dissimilar among treatments for the frequency range from 5 to 15 Hz (**Fig. 2.6A – D**). The radar plot (**Fig. 2.6E**) shows average EEG power in six frequency bands as percent change from saline (gray fill). Fentanyl (red) significantly (red asterisks) decreased EEG power in the theta, alpha, and sigma frequency ranges. Morphine (green) caused a significant (green asterisks) decrease in EEG power in the alpha and sigma frequencies. Descriptive statistics for **Fig. 2.6** are in **Supplemental Table S2.7**. F-values, numerator (n) and denominator (d) degrees of freedom (DF), P-values, and effect sizes (Eta squared) for the Fig. 2.6 results are reported in **Supplemental Table S2.8**.

Discussion

Three points shape the following discussion. First is the discovery that antinociceptive doses of fentanyl, morphine, and buprenorphine significantly and differentially disrupt the temporal organization of sleep and wakefulness. Such data from B6 mice were heretofore not available in the literature or in the Mouse Phenome Database (https://phenome.jax.org/). The present results contribute to community phenotyping efforts by making publicly available all descriptive and inferential statistics from this study (**Supplemental Tables S2.1** through **S2.8**). Second, opioids cause state dissociations by producing EEG slow waves during wakefulness that normally occur during NREM sleep. The final section highlights the relevance of these novel data for consciousness studies.

Opioids Disrupt the Temporal Organization of Sleep and Wakefulness

Opioid receptors modulate neuronal excitability by triggering a series of intracellular events that inhibit neuronal firing. Neuronal hyperpolarization occurs via inhibition of adenylyl cyclase, activation of potassium conductance out of neurons, and inhibition of calcium conductance into neurons which decreases presynaptic neurotransmitter release (James & Williams, 2020). All three opioids tested in this study modulate neuronal excitability by activating mu opioid receptors. However, there are pharmacokinetic and pharmacodynamic differences among these opioids, which led us to hypothesize differential effects of these opioids on sleep and EEG power. Fentanyl is highly lipid soluble, enters the brain rapidly, and has no active metabolites. Morphine has lower lipid solubility, resulting in slower uptake than fentanyl. Morphine has an active metabolite that is potent and long lasting. Fentanyl and morphine are full agonists at mu opioid receptors (Yaksh & Wallace, 2018). Fentanyl, but not morphine, also blocks alpha-1 adrenergic receptors and dopamine D4.4 and D1 receptors, and blocks reuptake of

neurotransmitters by the vesicular monoamine transporter2 (Torralva et al., 2020). Buprenorphine is highly lipid soluble and has long lasting metabolites with pharmacological activity. In contrast to fentanyl and morphine, buprenorphine is a partial mu opioid receptor agonist and a partial kappa opioid receptor agonist with low efficacy, thus acts as an antagonist at kappa opioid receptors (Huang et al., 2001).

The fact that opioid receptors modulate neuronal excitability (Winters et al., 2017) was used by the present study to characterize how systemically administered opioids alter the architecture of sleep and wakefulness. All three opioids disrupted the percent of time spent in each state of consciousness, the number of episodes, and episode duration (**Fig. 2.2**). These 4-h results led to a second series of experiments showing that the temporal organization of sleep and wakefulness was disrupted for up to 24 h after acute opioid administration (**Figs. 2.3 and 2.4**). Administering opioids during the initial hours of the light period caused a reduction in wakefulness during the mouse subjective night and increased sleep during the mouse subjective day. The number and duration of state episodes also varied as a function of the light:dark cycle and the specific opioid administered. Examining the time course of opioid effects (**Fig. 2.5**) showed wakefulness was shifted into the light phase, and that NREM sleep and REM sleep were shifted into the dark phase. Relative to fentanyl and morphine, buprenorphine caused the most severe disruption in the temporal organization of sleep and wakefulness, which is likely due, at least in part, to actions of the high affinity, long lasting metabolite, norbuprenorphine (Huang et al., 2001).

Opioids Cause a Dissociated State of Wakefulness and Alter EEG power

A practical way to define a behavioral state, or a state of consciousness, was put forth by Steriade and McCarley (Steriade & McCarley, 2005): "A state is a recurring, temporally enduring constellation of values of a set of indicator variables of the organism." States of sleep

and wakefulness are defined by a set of physiological values, or traits, that include EEG and EMG. Dissociated states of consciousness occur when a trait characteristic of one state (e.g., skeletal muscle tone during wakefulness) intrudes into another state (e.g., REM sleep). Dissociated states of consciousness are not rare in humans. NREM sleep parasomnias involve sleep intruding into wakefulness, as exemplified by confusional arousals, sleep terrors, sleepwalking, and sleep-related eating disorders (Castelnovo et al., 2018). REM sleep parasomnias, such as REM sleep behavior disorder, involve waking behaviors (e.g., speaking, complex motor activity) that intrude into sleep (Holtbernd et al., 2021; Schenck & Mahowald, 2002). In clinical sleep medicine, these types of parasomnias are classified as state dissociation disorders (Avidon, 2017).

Although EEG slow waves are normally associated with NREM sleep (Adamantidis et al., 2019), previous studies consistently show that opioids cause dissociated states of consciousness in which EEG slow waves appear during wakefulness. Opioid-induced wakefulness with EEG delta activity has been found in dog (Pickworth & Sharpe, 1979), cat (de Andres et al., 1984), rat (Osman et al., 2005), and human (Greenwald & Roehrs, 2005). The present results show that across 4 h of recording, fentanyl, morphine, and buprenorphine caused EEG delta activity during wakefulness. After saline administration during the light phase, sleep in mice is normally punctuated by brief episodes of wakefulness. EEG spectrograms (**Fig. 2.1**, right) show bouts of wakefulness as brief disruptions in the dark red band. In contrast, fentanyl, morphine, and buprenorphine each caused prolonged intervals of wakefulness with continuous EEG slow-wave activity. The presence of EEG slow-wave activity during wakefulness indicates that opioids cause a dissociated state of wakefulness in B6 mice. The results are limited by having tested only one dose of each opioid. Doses previously shown to be antinociceptive in mice were

selected for this study. The results encourage ongoing experiments to derive concentration-response data characterizing the effects of opioids on EEG power and states of consciousness (O'Brien et al., 2021; Zebadúa Unzaga et al., 2021). Future studies using female mice also are needed.

Measures of total EEG power that combine all frequency bands during wakefulness (**Fig. 2.6A-D**) showed that each opioid decreased total power within the 0 to 60 Hz range. Analyzing EEG by distinct frequency bands (**Fig. 2.6E**) revealed that fentanyl significantly decreased EEG power in the theta band. Previous studies of mice show that EEG theta is prominent during NREM sleep and REM sleep (Franken et al., 1998). During wakefulness, when EEG theta is also prominent (Vyazovskiy et al., 2009), fentanyl significantly decreased EEG theta. In B6 mice EEG frequency can be coupled to respiratory frequency (Tort et al., 2018). Fentanyl causes a dose-dependent decrease in respiratory rate in B6 mice (Fechtner et al., 2015). The present results and previous data from rat (Montandon & Horner, 2019) encourage studies in mice designed to quantify the extent to which respiratory rhythm modulated the fentanyl-induced decrease in EEG theta.

EEG power in alpha and sigma bands was significantly decreased by both morphine and fentanyl. In humans, EEG alpha activity occurs during the transition from a state of quiet wakefulness with eyes closed, to the state of NREM sleep (Carskadon & Dement, 2017). In mice, EEG alpha activity is pronounced during epochs of quiet wakefulness (Franken et al., 1998). The finding that fentanyl and morphine decreased alpha power during active wakefulness is consistent with evidence that opioids cause a blunting of wakefulness (Lydic et al., 2021). EEG sigma activity in B6 mice occurs during NREM sleep and sigma power increases before the onset of REM sleep (Vyazovskity et al., 2004). Consistent with the opioid-induced enhancement

of wakefulness and decrease in NREM sleep, EEG sigma power was significantly decreased by both morphine and fentanyl. Considered together, the results shown in **Figs. 2.2-2.6** are limited to opioids administered at a single time point during the light phase. The results encourage derivation of opioid-specific, phase-response curves for EEG power in B6 mice. Previous studies have shown that hamster wheel running activity is phase-shifted by fentanyl (Meijer et al., 2000).

Mechanistic studies (Adamantidis et al., 2019; Gent et al., 2018; Vyazovskiy et al., 2009) have extended to mice the classic findings of Steriade and colleagues (Steriade et al., 1993) that relate oscillating thalamo-cortical neuronal activity to the temporal distribution of sleep and wakefulness. The fundamental importance of rhythmic network excitability is emphasized by the in utero expression of respiratory movements (Niblock et al., 2020) and EEG rhythms (Rurak, 2016). Fetal human EEG underlies the construct of protoconsciousness, which is a proposed starting point for the development of adult consciousness (Hobson, 2009). In mice, measures of neonatal EEG across development show that rhythmic changes in brain excitability underlie the future expression of wakefulness, NREM sleep, and REM sleep (Rensing et al., 2018).

Opioids, Sleep, and Consciousness Studies

Sleep as a platform for consciousness studies is indisputable from philosophical (Windt, 2021), biological (Vyazovskiy et al., 2009), and healthcare (Kryger et al., 2021) perspectives. The fact that the phenomenology of consciousness is produced by the brain (Revonsuo, 2006) is further illustrated by this journal's invitation of papers on consciousness. An abundance of studies show that the neural correlates of consciousness are measurable entities, indicating that consciousness is not limited to being defined by its disruption or eradication. Regarding the biology of

consciousness, the genomic homology between human and non-human animals supports a widely shared view that studies of non-humans can contribute to understanding consciousness in humans. Proponents of affective neuroscience note that genomic homology supports the interpretation that non-human animals possess some form of primary-process consciousness (Panksepp, 2005). Finally, from a healthcare perspective, the buprenorphine-induced sleep disruption in B6 mice is homologous to human sleep disruption caused by buprenorphine used in medication assisted therapy for opioid use disorder (Dunn et al., 2018; Farney et al., 2013; Garnaat et al., 2017). Pain is also an altered state of consciousness (Garcia-Larrea & Bastuji, 2018) and sleep disruption increases pain sensitivity in humans (Roehrs et al., 2006), rats (Vanini et al., 2014), and mice (Alexandre et al., 2017). Thus, opioids, pain, and sleep modulate interacting states of consciousness. The potential clinical significance of this interaction is instantiated by evidence that disordered sleep in humans is a risk factor for addiction relapse (Brower & Perron, 2010; Valentino & Volkow, 2020).

Acknowledgements

Gratefully acknowledge support from the University of Tennessee. For technical assistance we thank Carolina Bustamante, Megan Thibert, and Wilton Sun.

References

Adamantidis, A. R., Gutierrez Herrera, C., & Gent, T. C. (2019). Oscillating circuitries in the sleeping brain. *Nature Reviews Neuroscience, 20*(12): 746-762. https://doi.org/10.1038/s41583-019-0223-4

Alexandre, C., Latremoliere, A., Ferreira, A., Miracca, G., Yamamoto, M., Scammell, T. E., & Woolf, C. J. (2017). Decreased alertness due to sleep loss increases pain sensitivity in mice. *Nat Med*, *23*: 768-774. https://doi.org/10.1038/nm.4329

Angel, T. C., Glovak, A. Z., Alami, A. W., Mihalko, A. S., Price, A. J., Jiang, A. Y., Baghdoyan, H. A., & Lydic, A. R. (2018). Buprenorphine depresses respiratory variability in obese mice with altered leptin signaling. *Anesthesiology, 128*(5): 984-991. https://doi.org/10.1097/ALN.0000000000002073

Aserinsky, E., & Kleitman, N. (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. *Science, 118*(3062): 273-274. http://www.jstor.org.utk.idm.oclc.org/stable/1680525

Avidon, A. Y. (2017). Non-rapid eye movement parasomnias: Clinical spectrum, diagnostic features, and management. In M. Kryger, T. Roth, & W. C. Dement (Eds.), *Principles and practice of sleep medicine, sixth edition* (Sixth ed., pp. 981-992). Elsevier.

Brower, K. J., & Perron, B. E. (2010). Sleep disturbance as a universal risk factor for relapse in addictions to psychoactive substances. *Medical Hypotheses*, *74*(5): 928-933. https://doi.org/10.1016/j.mehy.2009.10.020

Carskadon, M. A., & Dement, W. C. (2017). Normal human sleep: An overview. In M. H. Kryger, T. Roth, & W. C. Dement (Eds.), *Principles and practice of sleep medicine, sixth edition* (6th ed., pp. 15-24). Elsevier.

Castelnovo, A., Lopez, R., Proserpio, P., Nobili, L., & Dauvilliers, Y. (2018). Nrem sleep parasomnias as disorders of sleep-state dissociation. *Nat Rev Neurol., 14*: 470-481. https://doi.org/10.1038/s41582-018-0030-y

Charbogne, P., Kieffer, B. L., & Befort, K. (2014). 15 years of genetic approaches in vivo for addiction research: Opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology, 76*: 204-217. https://doi.org/10.1016/j.neuropharm.2013.08.028

de Andres, I., Villablanca, J. R., & Burgess, J. W. (1984). Reassessing morphine effects in cats: Ii. Protracted effects on sleep-wakefulness and the eeg. *Pharmacology Biochemistry and Behavior, 21*(6): 923-928. https://doi.org/10.1016/S0091-3057(84)80074-X

Dement, W. C. (1958). The occurrence of low voltage, fast, electroencephalogram patterns during behavioral sleep in the cat. *EEG Clin Neurophysiol, 10*: 291-296. https://doi.org/10.1016/0013-4694(58)90037-3

Dement, W. C., & Kleitman, N. (1957). Cyclic variations in eeg during sleep and their relation to eye movements, body motility and dreaming. *EEG Clin Neurophysiol, 9*: 673-690. https://doi.org/10.1016/0013-4694(57)90088-3

Dunn, K. E., Finan, P. H., Tompkins, A., & Strain, E. C. (2018). Frequency and correlates of sleep disturbance in methadone and buprenorphine-maintained patients. *Addict Behav, 76*: 8-14. https://doi.org/10.1016/j.addbeh.2017.07.016

Farney, R. J., McDonald, A. M., Boyle, K. M., Snow, G. L., Nuttall, R. T., Coudreaut, M. F., Wander, T. J., & Walker, J. M. (2013). Sleep disordered breathing in patients receiving therapy with buprenorphine/naloxone. *Eur Respir J, 42*: 394-403. https://doi.org/10.1183/09031936.00120012

Fechtner, L., Ali, M. E., Sattar, A., Moore, M., & Strohl, K. P. (2015). Fentanyl effects on breath generation in c57bl/6j and a/j mouse strains. *Respiratory Physiology & Neurobiology, 215*: 20-29. https://doi.org/10.1016/j.resp.2015.04.011

Flint, R. R., Chang, T., Lydic, R., & Baghdoyan, H. A. (2010). Gaba-a receptors in the pontine reticular formation of c57bl/6j mouse modulate neurochemical, electrographic, and behavioral phenotypes of wakefulness. *The Journal of Neuroscience, 30*(37): 12301-12309. https://doi.org/10.1523/jneurosci.1119-10.2010

Franken, P., Malafosse, A., & Tafti, M. (1998). Genetic variation in eeg activity during sleep in inbred mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 275*(4): R1127-R1137. https://doi.org/10.1152/ajpregu.1998.275.4.R1127

Gades, N. M., Danneman, P. J., Wixson, S. K., & Tolley, E. A. (2000). The magnitude and duration of the analgesic effect of morphine, butorphanol, and buprenorphine in rats and mice. *Contemp Top Lab Anim Sci, 39*(2): 8-13.

Garcia-Larrea, L., & Bastuji, H. (2018). Pain and consciousness. *Prog Neuropsychopharmacol Biol Psychiatry, 87*: 193-199. https://doi.org/10.1016/j.pnpbp.2017.10.007

Garnaat, S. L., Weisberg, R. B., Uebelacker, L. A., Herman, D. S., Bailey, G. L., Anderson, B. J., Sharkey, K. M., & Stein, M. D. (2017). The overlap of sleep disturbance and depression in primary care patients treated with buprenorphine. *Subst Abus, 38*: 450-454. https://doi.org/10.1080/08897077.2017.1361498

Gent, T. C., Bandarabadi, M., Gutierrez Herrera, C., & Admantidis, A. R. (2018). Thalamic dual control of sleep and wakefulness. *Nature Neuroscience, 21*: 974-984. https://doi.org/10.1038/s41593-018-0164-7

Greenwald, M. K., & Roehrs, T. A. (2005). Mu-opioid self-administration vs passive

administration in heroin abusers produces differential eeg activation. *Neuropsychopharmacology*, *30*(1): 212-221. https://doi.org/10.1038/sj.npp.1300596

Hobson, J. A. (2009). Rem sleep and dreaming: Towards a theory of protoconsciousness. *Nature Reviews. Neuroscience*, *10*: 803-812. https://doi.org/10.1038/nrn2716

Holtbernd, F., Romanzetti, S., Oertel, W. H., Knake, S., Sittig, E., Heidbreder, A., Maier, A., Krahe, J., Wojtala, J., Dogan, I., Schultz, J. B., Schiefer, J., Janzen, A., & Reetz, K. (2021). Convergent patterns of structural brain changes in rapid eye movement sleep behavior disorder and parkinson's disease on behalf of the german rapid eye movement sleep behavior disorder study group. *Sleep*, *44*(3): 1-12. https://doi.org/10.1093/sleep/zsaa199

Huang, P., Kehner, G. B., Cowan, A., & Liu-Chen, L.-Y. (2001). Comparison of pharmacological activities of buprenorphine and norbuprenorphine: Norbuprenorphine is a potent opioid agonist. *The Journal of Pharmacology and Experimental Therapeutics*, *297*(2): 688-695.

James, A., & Williams, J. (2020). Basic opioid pharmacology - an update. *Br J Pain*, *14*(2): 1115-1121. https://doi.org/10.1177/2049463720911986

Kryger, M. H., Roth, T., & Dement, W. C. (Eds.). (2021). *Principles and practice of sleep medicine, seventh edition* (7th ed.). Elsevier.

Locklear, C. E., Bustamante, C. G., Glovak, Z. T., Zebadúa Unzaga, D., O'Brien, C. O., Lydic, R., & Baghdoyan, H. A. (2019). Systemically administered opiates disrupt the architecture of sleep and wakefulness in mouse. *Neuroscience Meeting Planner*, *502.02*. https://www.abstractsonline.com/pp8/#!/7883/presentation/54154

Loomis, A. L., Harvey, E. N., & Hobart, G. (1935). Potential rhythms of the cerebral cortex during sleep. *Science*, *81*: 597-598. https://www.jstor.org/stable/1659223

Lutfy, K., Eitan, S., Bryant, C. D., Yang, Y. C., Saliminejad, N., Walwyn, W., Kieffer, B. L.,

Takeshima, H., Carroll, F. I., Maidment, N. T., & Evans, C. J. (2003). Buprenorphine-induced antinociception is mediated by mu-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. *The Journal of Neuroscience, 23*: 10331-10337.

Lutz, P.-E., & Kieffer, B. L. (2013). The multiple facets of opioid receptor function: Implications for addiction. *Current Opinion in Neurobiology, 23*(4): 473-479. https://doi.org/10.1016/j.conb.2013.02.005

Lydic, R., & Baghdoyan, H. A. (2021). Prefrontal cortex metabolome is modified by opioids, anesthesia, and sleep. *Physiology, 36*(4): 203-209. https://doi.org/10.1152/physiol.00043.2020

Lydic, R., Hillman, D. R., Jiang, Y., Baghdoyan, H. A., & O'Brien, C. O. (2021). Opioid actions on sleep and breathing. In M. H. Kryger, T. Roth, & W. C. Dement (Eds.), *Principles and practice of sleep medicine, seventh edition* (7th ed., pp. In Press). Elsevier.

Meijer, J. H., Ruijs, A. C. J., Albus, H., van de Geest, B., Duindam, H., Zwinderman, A. K., & Dahan, A. (2000). Fentanyl, a mu-opioid receptor agonist, phase shifts the hamster circadian pacemaker. *Brain Research, 2000*: 135-140. https://doi.org/10.1016/S0006-8993(00)02317-9

Mitler, M. M., Lund, R., Sokolove, P. G., Pittendrigh, C. S., & Dement, W. C. (1977). Sleep and activity rhythms in mice: A description of circadian patterns and unexpeted disruptions of sleep. *Brain Research, 131*: 129-145. https://doi.org/10.1016/0006-8993(77)90033-6

Monaco, G., van Dam, S., Ribeiro, J. L. C. N., Larbi, A., & de Magalhães, J. P. (2015). A comparison of human and mouse gene co-expression networks reveals conservation and divergence at the tissue, pathway and disease levels. *BMC Evol Biol, 15:259*: 1-14.

https://doi.org/10.1186/s12862-015-0534-7

Montandon, G., & Horner, R. L. (2019). Electrocortical changes associating sedation and respiratory depression by the opioid analgesic fentanyl. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-50613-2

Mouse Genome Sequencing Consortium. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, *420*: 520-562. https://doi.org/10.1038/nature01262

Niblock, M. M., Perez, A., Broitman, S., Jacoby, B., Aviv, E., & Gilkey, S. (2020). In utero development of fetal breathing movements in c57bl6 mice. *Respiratory Physiology & Neurobiology*, *271*(103288): 1-9. https://doi.org/10.1016/j.resp.2019.103288

O'Brien, C. B., Zebadúa Unzaga, D., Lydic, R., & Baghdoyan, H. A. (2021). Fentanyl, but not morphine, increases cortical electroencephalographic (EEG) delta power during wakefulness in C57BL/6J mice. *FASEB Journal*, *35*. https://doi.org/10.1096/fasebj.2021.35.S1.01699

O'Brien, C. B., Baghdoyan, H. A., & Lydic, R. (2019). Computer-based multitaper spectrogram program for electroencephalographic data. *J. Vis. Exp.*, *153*(e60333): 1-7. https://doi.org/10.3791/60333

Osman, I., Nadir, Baghdoyan, H. A., & Lydic, R. (2005). Morphine inhibits acetylcholine release in rat prefrontal cortex when delivered systemically or by microdialysis to basal forebrain. *Anesthesiology*, *103*(4): 779-787. https://doi.org/10.1097/00000542-200510000-00016

Panksepp, J. (2005). Affective consciousness: Core emotional feelings in animals and humans. *Conscious Cogn, 14*: 30-80. https://doi.org/10.1016/j.concog.2004.10.004

Paxinos, G., & Franklin, K. B. J. (2019). *The mouse brain in stereotaxic coordinates* (5th ed.). Academic Press.

Pickworth, W. B., & Sharpe, L. G. (1979). Eeg-behavioral dissociation after morphine and cyclazocine-like drugs in the dog: Further evidence for two opiate receptors. *Neuropharmacology*, *18*(7): 617-622. https://doi.org/10.1016/0028-3908(79)90114-X

Pontius, J. U., Mullikin, J. C., Smith, D. R., Lindblad-Toh, K., Gnerre, S., Clamp, M., Chang, J., Stephens, R., Neelam, B., Volfovsky, N., Schaffer, A. A., Agarwala, R., Narfstrom, K., Murphy, W. J., Giger, U., Roca, A. L., Antunes, A., Menotti-Raymond, M., Yuhki, N., . . . O'Brien, S. J. (2007). Initial sequence and comparative analysis of the cat genome. *Genome Res*, *17*(11): 1675-1689. https://doi.org/10.1101/gr.6380007

Prerau, M. J., Brown, R. E., Bianchi, M. T., Ellenbogen, J. M., & Purdon, P. L. (2017). Sleep neurophysiological dynamics through the lens of multitaper spectral analysis. *Physiology (Bethesda, Md.)*, *32*(1): 60-92. https://doi.org/10.1152/physiol.00062.2015

Ramirez, J.-M., Burgraff, N. J., Wei, A. D., Baertsch, N. A., Varga, A. G., Baghdoyan, H. A., Lydic, R., Morris, K. F., Bolser, D. C., & Levitt, E. S. (2021). Neuronal mechanisms underlying opioid-induced respiratory depression: Our current understanding. *Journal of Neurophysiology*, *125*(5): 1899-1919. https://doi.org/10.1152/jn.00017.2021

Rensing, N., Moy, B., Friedma, J. L., Galindo, R., & Wong, M. (2018). Longitudinal analysis of developmental changes in electroencephalography patterns and sleep-wake states of the neonatal mouse. *PLoS ONE*, *13*(e0207031): 1-17. https://doi.org/10.1371/journal.pone.0207031

Revonsuo, A. (2006). *Inner presence*. MIT Press.

Richardson, G. S., Moore-Ede, M. C., Czeisler, C. A., & Dement, W. C. (1985). Circadian rhythms of sleep and wakefulness in mice: Analysis using long-term automated recording of sleep. *Am. J. Physiol.*, *248*: R320-R330. https://doi.org/10.1152/ajpregu.1985.248.3.R320

Roehrs, T., Hyde, M., Blaisdell, B., Greenwald, M., & Roth, T. (2006). Sleep loss and rem sleep loss are hyperalgesic. *Sleep, 29*(2): 145-151. https://doi.org/10.1093/sleep/29.2.145

Rurak, D. (2016). Fetal sleep and spontaneous behavior in utero: Animal and clinical studies. In D. W. Walker (Ed.), *Prenatal and postnatal determinants of development* (pp. 89-146). Humana Press. https://doi.org/10.1007/978-1-4939-3014-2_6

Schenck, C. H., & Mahowald, M. W. (2002). Rem sleep behavior disorder: Clinical, developmental, and neuroscience perspectives 16 years after its formal identification. *Sleep, 25*: 120-138. https://doi.org/10.1093/sleep/25.2.120

Steriade, M., & McCarley, R. W. (2005). *Brain control of wakefulness and sleep* (Second ed.). Kluwer Academic/Plenum Publishers.

Steriade, M., McCormick, D. A., & Sejnowski, T. J. (1993). Thalamocortical oscillations in the sleeping and aroused brain. *Science, 262*: 679-685. https://doi.org/10.1126/science.8235588

Torralva, R., Eshleman, A. J., Swanson, T. L., Schmachtenberg, J. L., Schutzer, W. E., Bloom, S. H., Wolfrum, K. M., Reed, J. F., & Janowsky, A. (2020). Fentanyl but not morphine interacts with nonopioid recombinant human neurotransmitter receptors and transporters. *Journal of Pharmacology and Experimental Therapeutics*, *374*(3): 376-391. https://doi.org/10.1124/jpet.120.265561

Tort, A., Ponsel, S., Jessberger, J., Yanovsky, Y., Brankačk, J., & Draguhm, A. (2018). Parallel detection of theta and respiration-coupled oscillations throughout the mouse brain. *Scientific Reports, 8*: 1-14. https://doi.org/10.1038/s41598-018-24629-z

Valatx, J. L., Bugat, R., & Jouvet, M. (1972). Genetic studies of sleep in mice. *Nature*, *238*(5361): 226-227. https://doi.org/10.1038/238226a0

Valentino, R. J., & Volkow, N. D. (2020). Drugs, sleep, and the addicted brain.

Neuropsychopharmacology, *45*(1): 3-5. https://doi.org/10.1038/s41386-019-0465-x

Vanini, G., Nemanis, K., Baghdoyan, H. A., & Lydic, R. (2014). Gabaergic transmission in rat pontine reticular formation regulates the induction phase of anesthesia and modulates hyperalgesia caused by sleep deprivation. *European Journal of Neuroscience*, *40*(1): 2264-2273. https://doi.org/10.1111/ejn.12571

Varga, A. G., Maletz, S. N., Bateman, J. T., Reid, B. T., & Levitt, E. S. (2020). Neurochemistry of the kolliker-fuse nucleus from a respiratory perspective. *J Neurochem, 598.1*: 1-22. https://doi.org/10.1111/jnc.15041

Vyazovskity, V. V., Achermann, P., Borbély, A. A., & Tobler, I. (2004). The dynamics of spindles and eeg slow-wave active in nrem sleep in mice. *Arch Ital Biol, 142*: 511-523.

Vyazovskiy, V. V., Olcese, U., Lazimy, Y. M., Faraguna, U., Esser, S. K., Williams, J. C., Cirelli, C., & Tononi, G. (2009). Cortical firing and sleep homeostasis. *Neuron, 63*: 865-878. https://doi.org/10.1016/j.neuron.2009.08.024

Wimer, R. E., Wimer, C. C., & Roderick, T. H. (1969). Genetic variability in forebrain structures between inbred strains of mice. *Brain Research, 16*: 257-264. https://doi.org/10.1016/0006-8993(69)90098-5

Windt, J. M. (2021). How deep is the rift between conscious states in sleep and wakefulness? Spontaneous experience over the sleep–wake cycle. *Phil Trans R Soc B, 376*: 1-18. https://doi.org/10.1098/rstb.2019.0696

Winters, B. L., Gregoriou, G. C., Kissiwaa, S. A., Wells, O. A., Medagoda, D. I., Hermes, S. M., Burford, N. T., Alt, A., Aicher, S. A., & Bagley, E. A. (2017). Endogenous opioids regulate moment-to-moment neuronal communication and excitability. *Nature Communications, 8, 14611*: 1-15. https://doi.org/10.1038/ncomms14611

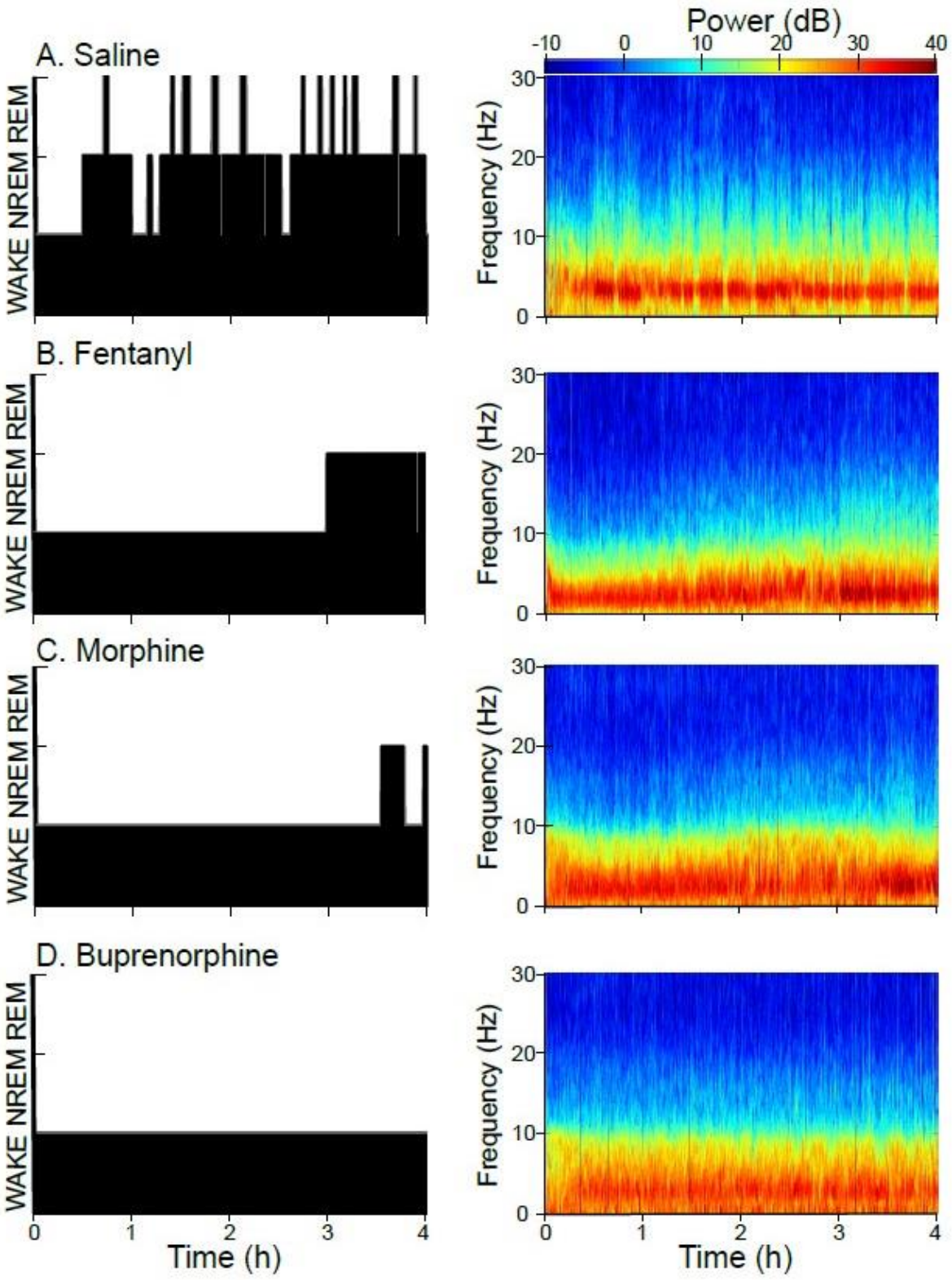
Yaksh, T., & Wallace, M. (2018). Opioids, analgesia, and pain management. In L. L. Brunton, R. Hilal-Dandan, & B. C. Knollmann (Eds.), *Goodman & gilman's the pharmacological basis of therapeutics, thirteenth edition* (pp. 355-386). McGraw Hill Education.

Zebadúa Unzaga, D., Bustamante, C., Thibert, M., & Baghdoyan, H. A. (2021). Fentanyl and morphine cause dose-dependent sleep disruption in c57bl/6j (b6) mice. *The FASEB Journal*, *35*. https://doi.org/10.1096/fasebj.2021.35.S1.01706

Zhang, X., Baer, A. G., Price, J. M., Jones, P. C., Garcia, B. J., Romero, J., Cliff, A. M., Mi, W., Brown, J. B., Jacobson, D. A., Lydic, R., & Baghdoyan, H. A. (2020). Neurotransmitter networks in mouse prefrontal cortex are reconfigured by isoflurane anesthesia. *Journal of Neurophysiology*, *123*(6): 2285-2296. https://doi.org/10.1152/jn.00092.2020

Appendix

Figure 2.1. Opioids disrupt the temporal organization of sleep and wakefulness and alter EEG power.
Representative time-course plots show sleep architecture **(left column)** and corresponding multi-taper spectrograms show EEG frequency (Hz) and EEG power (dB) **(right column)**. These 4-h recordings are from the same mouse after systemic administration on different days of saline (*A*), fentanyl (*B*), morphine (*C*), and buprenorphine (*D*).


A. Saline
B. Fentanyl
C. Morphine
D. Buprenorphine
WAKE NREM REM
Power (dB)
-10
0
10
20
30
40
Frequency (Hz)
0
10
20
30
0
1
2
3
4
Time (h)

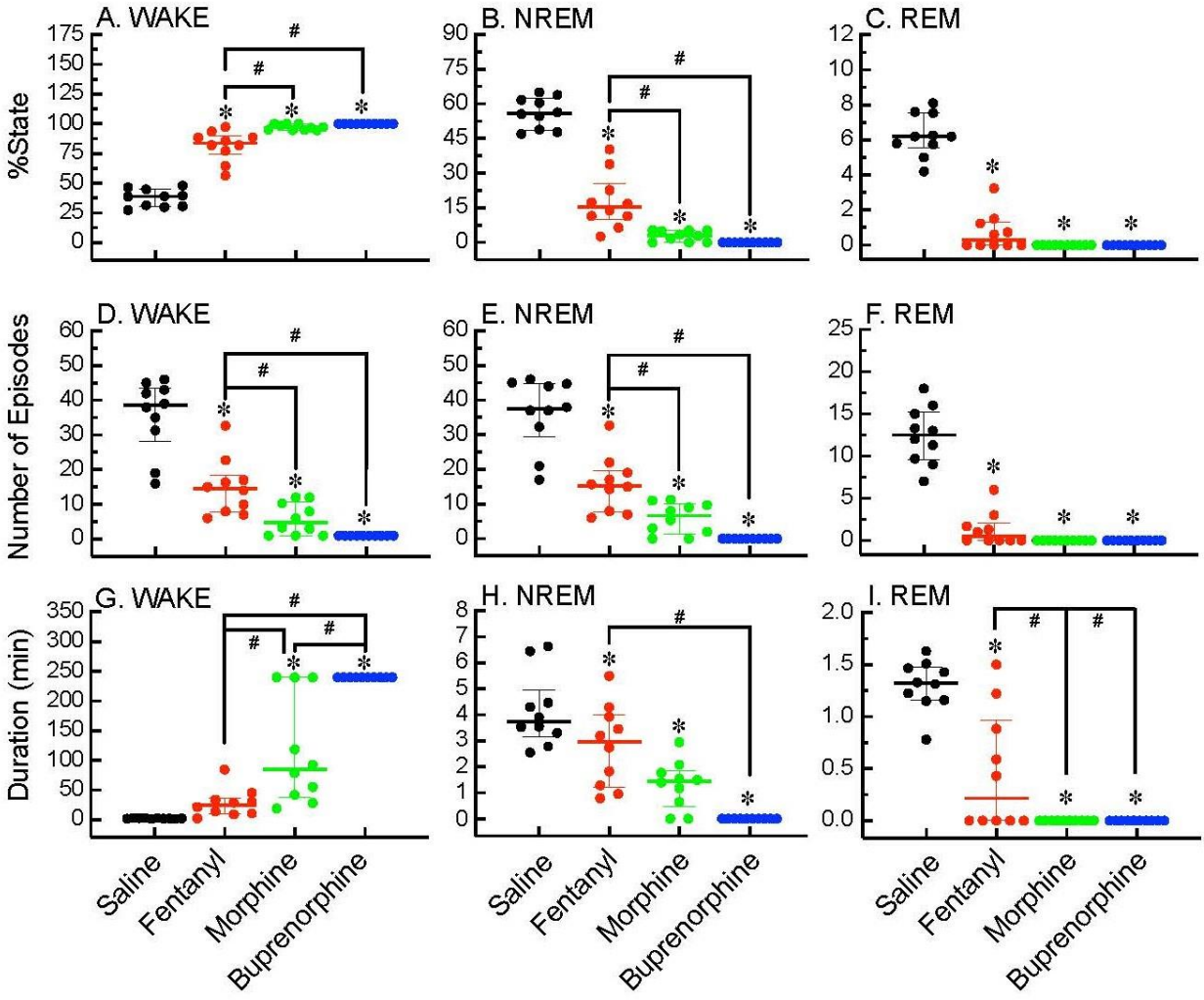
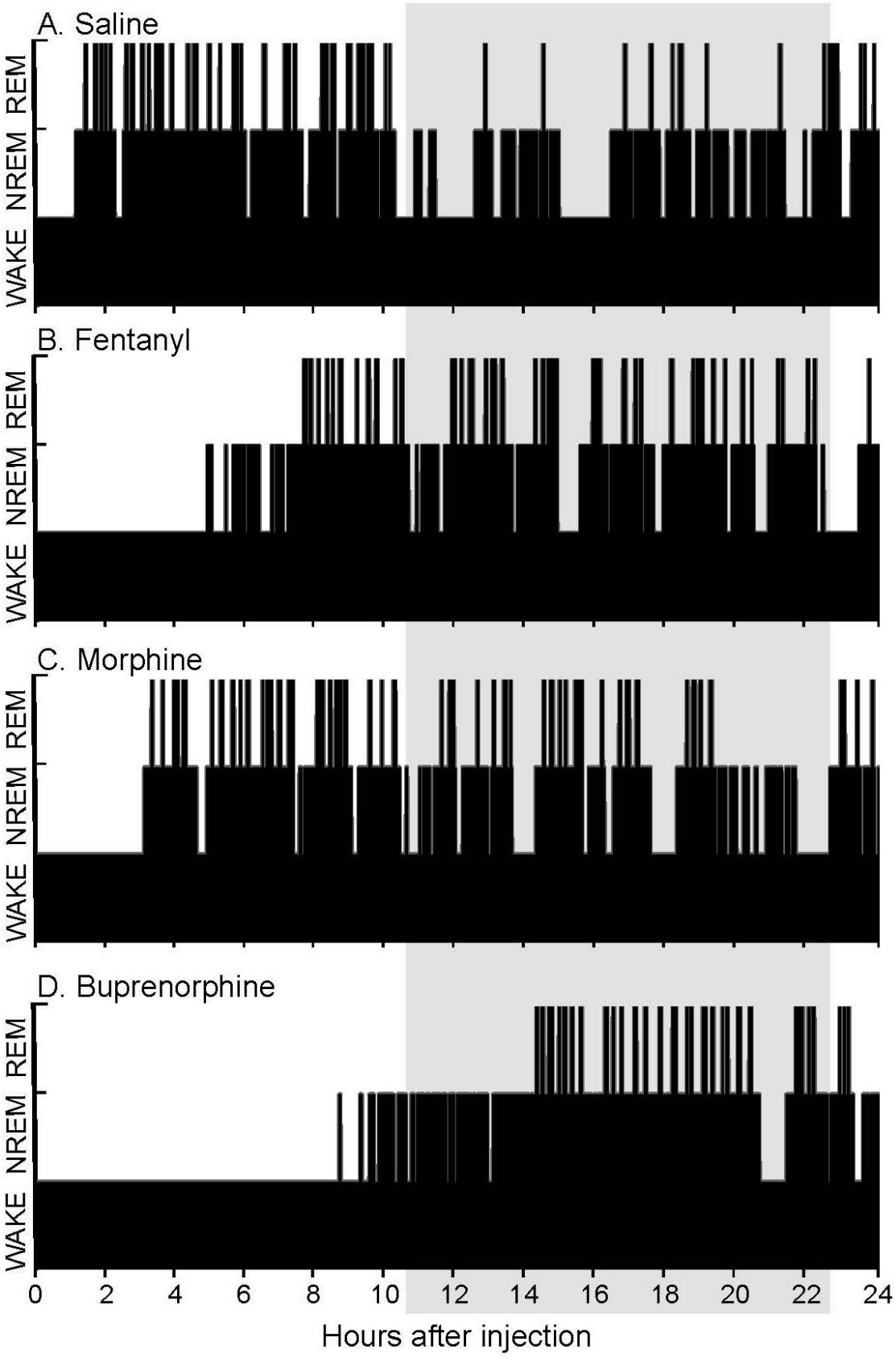


Figure 2.2. Systemic administration of opioids significantly disrupted sleep in B6 mice (n = 10).

For each injection condition (abscissa) the graphs summarize median and interquartile range of dependent measures during states of wakefulness (left column), NREM sleep (middle column), and REM sleep (right column). The top row shows percent of 4-h recordings in each state (A-C). The middle row plots the number of episodes for each state (D-F). The bottom row illustrates episode duration (G-I). In every panel, each dot represents data from one mouse. Data were analyzed by repeated measures one-way ANOVA and Tukey's multiple comparisons test. Asterisks indicate significant differences from saline, and number signs with brackets indicate significant differences between opioid effects. All 27 opioid-by-state measures were significantly different from saline. Significant differences between opioid effects were found for all dependent measures of wakefulness (A, D, and G) and NREM sleep (B, E, and H), and only for duration of REM sleep (I).

Figure 2.3. The temporal organization of sleep and wakefulness was disrupted for up to 24 h after systemic opioid administration (time 0).
These histograms from a single mouse are representative of the group data. The gray screen represents the 12-h dark period of the 12-h:12-h light:dark cycle during which sleep and wakefulness were recorded.


A. Saline
REM
NREM
WAKE
B. Fentanyl
REM
NREM
WAKE
C. Morphine
REM
NREM
WAKE
D. Buprenorphine
REM
NREM
WAKE
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours after injection

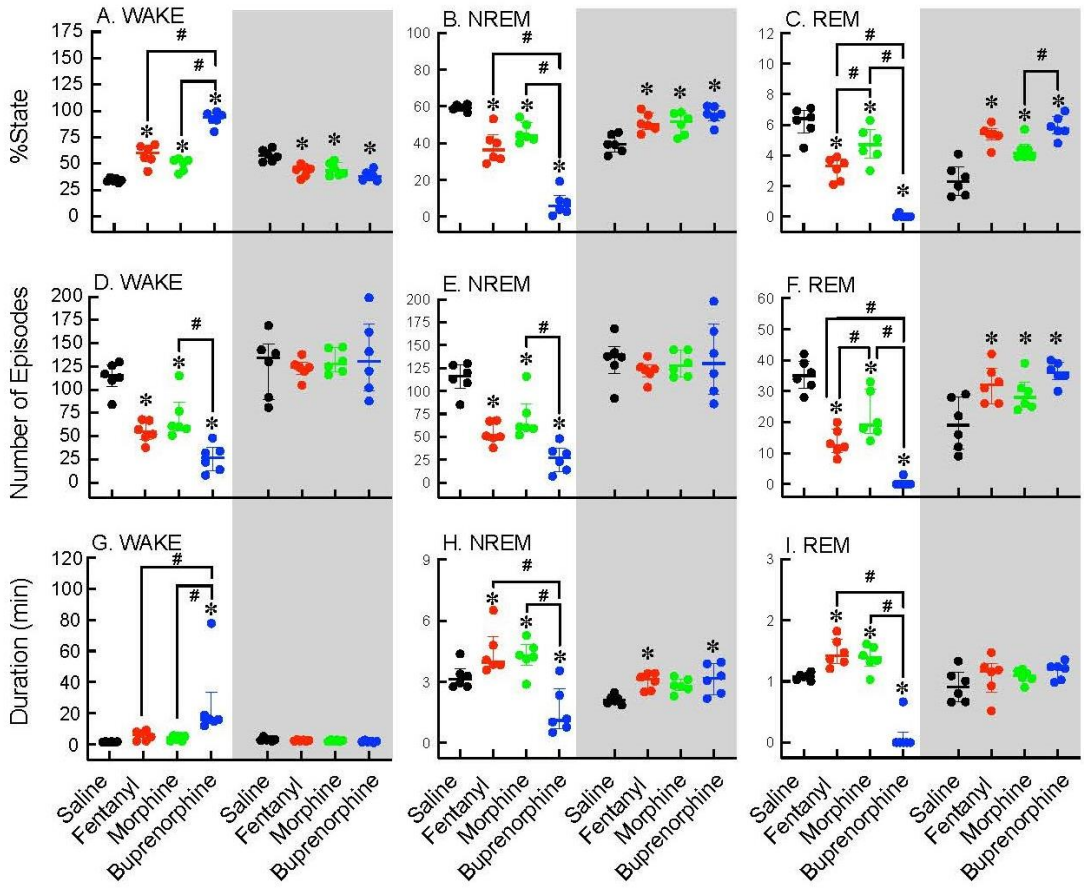


Figure 2.4. A single opioid injection significantly altered percent of time spent in wakefulness, NREM sleep, and REM sleep and sleep architecture.
A single opioid injection significantly altered the percent of time spent in wakefulness, NREM sleep, and REM sleep (top row), as well as the number (middle row) and duration (bottom row) of sleep and waking episodes for 24 h. The results from 6 mice recorded during the light period (no screen) and dark period (gray screen) are expressed as median and interquartile range. Each dot represents data from one mouse. Data were analyzed by repeated measures, two-way ANOVAs and Tukey's multiple comparisons tests. Asterisks indicate significant, opioid-induced changes from saline. Number signs and brackets summarize statistically significant differences between opioid effects.

Figure 2.5. Time course of differential opioid effects on states of consciousness.
Radar plots show the percent of time spent in wakefulness (A), NREM sleep (B), and REM sleep (C) averaged for 6 mice across 24 h. Key at top right identifies injection of saline (black), fentanyl (red), morphine (green), and buprenorphine (blue). Time after injection (hour 0) advances radially in a clockwise manner. The percent of time spent in each state (%) is plotted for each injection condition from high to low along the vertical line below hour 1. The saline (control) condition is illustrated by a gray fill. The dark phase of the light:dark cycle is designated by the black C-shaped bar on each radar plot. Note that at hour 1, all mice spent 100 percent of the time in a state of wakefulness (A), and zero percent time in NREM (B) or REM (C) sleep after opioid administration. Two-way ANOVAs and Tukey's multiple comparison tests revealed significant state main effects, time main effects, and a time-by-state interactions. For latency to onset of NREM sleep (D) and REM sleep (E), one-way ANOVAs revealed significant differences between saline and opioids (asterisks). Tukey's post-hoc comparisons indicated a significant difference between opioids (number signs above brackets).

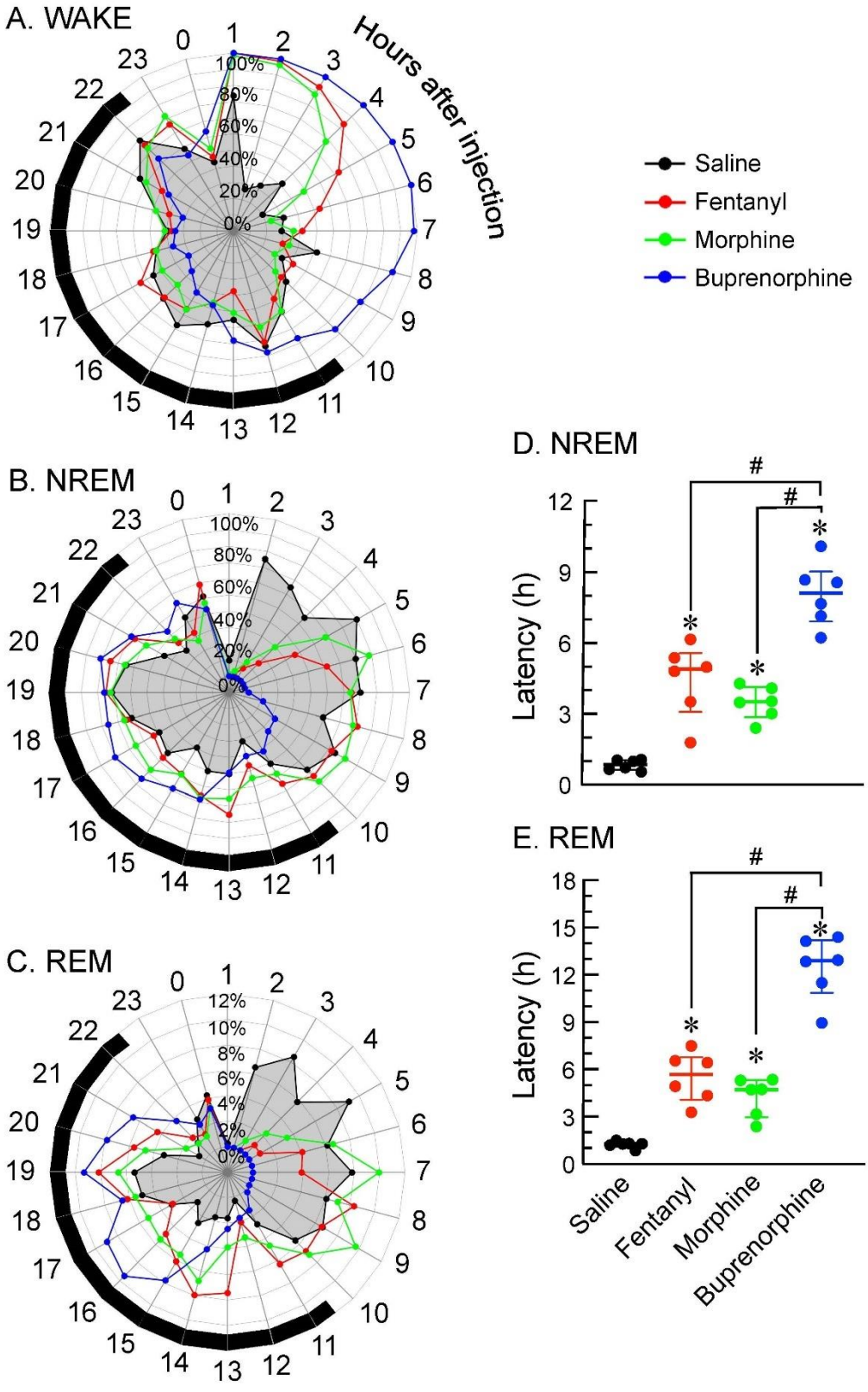
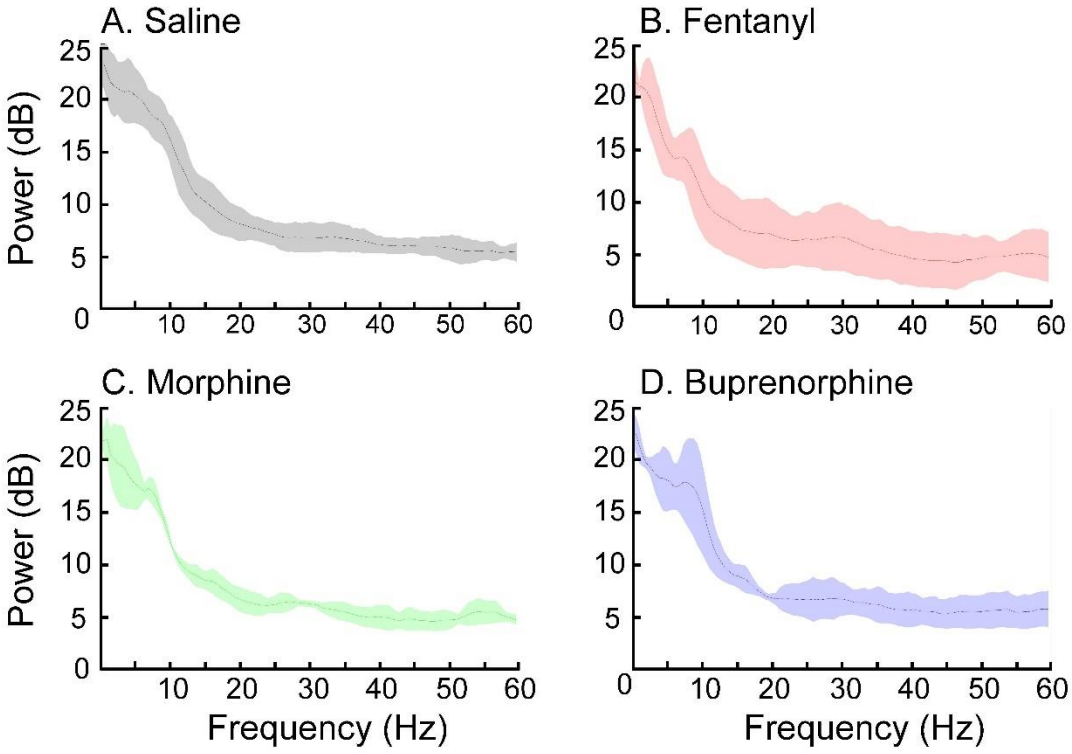
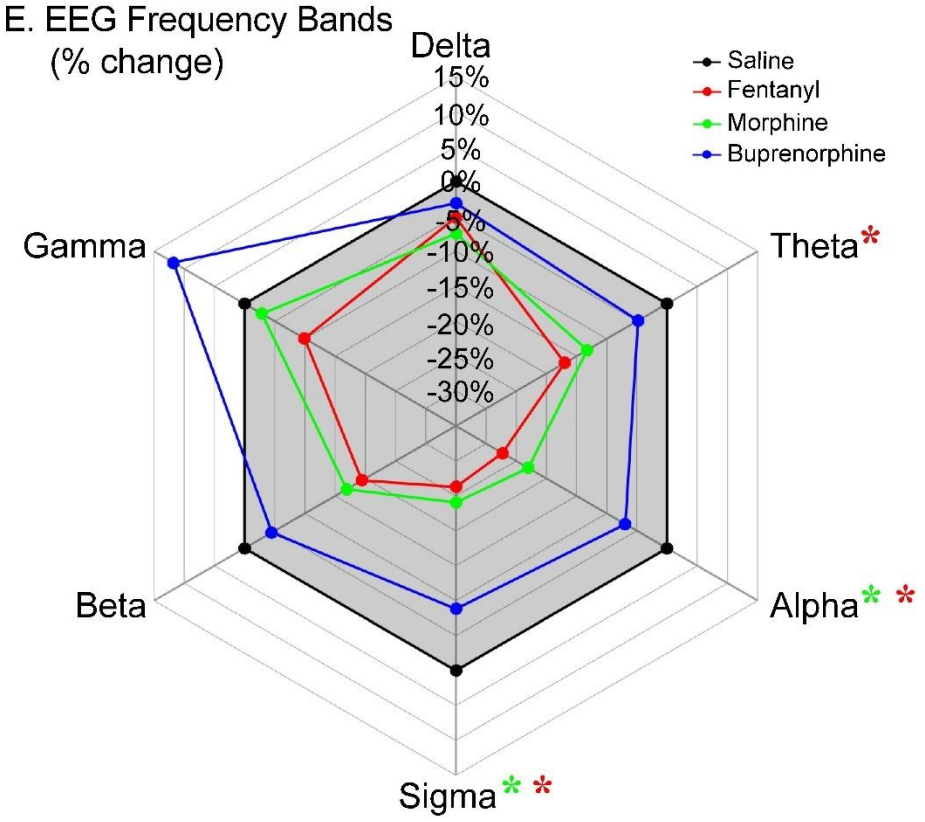

A. WAKE
Hours after injection
100%
80%
60%
40%
20%
0%
Saline
Fentanyl
Morphine
Buprenorphine
B. NREM
C. REM
12%
10%
8%
6%
4%
2%
D. NREM
Latency (h)
E. REM
Saline
Fentanyl
Morphine
Buprenorphine

Figure 2.6. EEG power was differentially altered by opioids during waking consciousness. EEG power was evaluated during the first 30 min after injection of saline (*A*), fentanyl (*B*), morphine (*C*) and buprenorphine (*D*). *A* – *D* plot spectral power (dB) as median (solid line) and interquartile range (shaded area) across frequencies ranging from 0 to 60 Hz. The radar plot (*E*) depicts opioid-specific EEG power as percent change from saline (gray fill). The key at top right of *E* shows the color code used for saline and each opioid. The 6 radial arms of the radar plot represent 6 different EEG frequency bands: delta (0.5 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 13 Hz), sigma (12 to 15 Hz), beta (13 to 30 Hz), and gamma (30 to 60 Hz). Data from 10 mice were analyzed by repeated measures one-way ANOVAs and Tukey's multiple comparisons tests. Asterisks indicate significant differences from saline and are color coded according to which opioid caused a significant effect.


A. Saline
B. Fentanyl
C. Morphine
D. Buprenorphine
Power (dB)
Frequency (Hz)
0
5
10
15
20
25
10
20
30
40
50
60


E. EEG Frequency Bands
(% change)
Delta
Theta*
Alpha* *
Sigma* *
Beta
Gamma
Saline
Fentanyl
Morphine
Buprenorphine
15%
10%
5%
0%
-5%
-10%
-15%
-20%
-25%
-30%

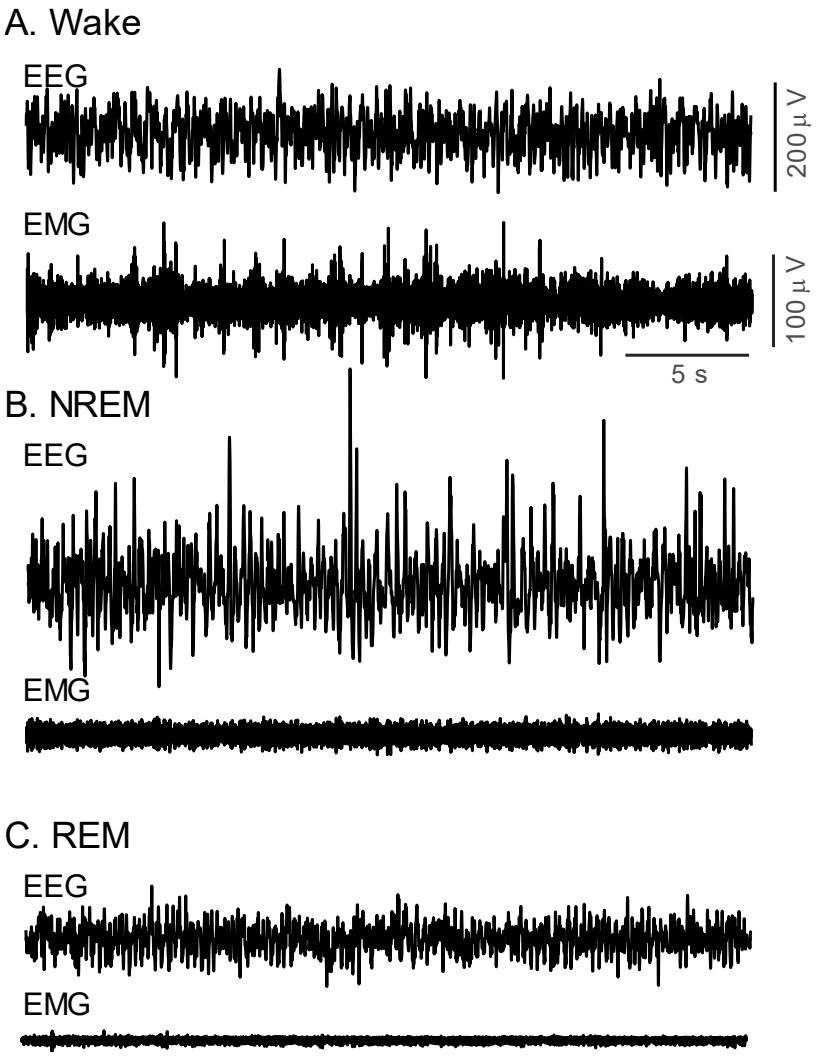
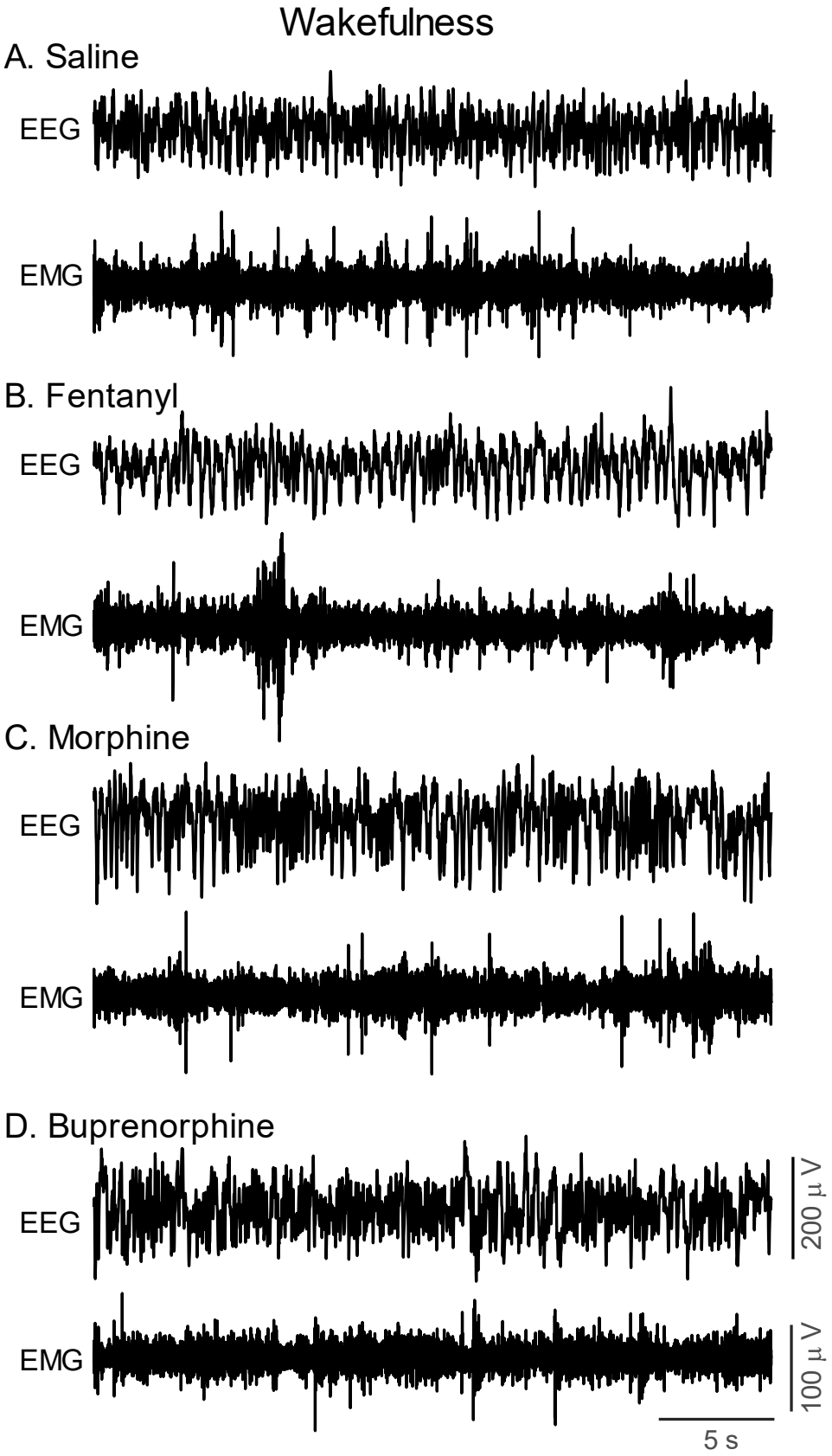


Figure 2.7. Representative, control recordings of the EEG and EMG.
Representative, control recordings of the cortical electroencephalogram (EEG) and electromyogram (EMG) illustrate signals used to visually identify and quantify states of wakefulness (A. Wake), non-rapid eye movement sleep (B. NREM), and rapid eye movement sleep (C. REM) in adult, C57BL/6J (B6) mice. These recordings are from the same mouse used for the Fig. 2.1A histograms and Fig. 2.1B spectrograms, obtained after saline administration. Signals were filtered at 0.3 to 30 Hz for EEG, and at 10 to 100 Hz for EMG, with a 60 Hz notch filter used to reduce electrical noise.

Figure 2.8. Representative, recordings of the EEG and EMG during wakefulness 10 min post-injection.
Representative recordings of the cortical electroencephalogram (EEG) and electromyogram (EMG) were obtained during wakefulness, 10 min after intraperitoneal injection of (A) saline (vehicle control), (B) fentanyl, (C) morphine, and (D) buprenorphine. Saline and each of the opioids were administered on different days, with a minimum of one week between injections. Signals are from the same mouse shown Fig. 2.1 and Fig. 2.7. Comparison with the Fig. 2.7 recordings illustrates that wakefulness after opioid administration can be clearly identified by visual inspection of the EEG and EMG signals and cannot be mistaken for either stage of sleep. Signals were filtered at 0.3 to 30 Hz for EEG, and at 10 to 100 Hz for EMG, with a 60 Hz notch filter used to reduce electrical noise.


Wakefulness
A. Saline
EEG
EMG
B. Fentanyl
EEG
EMG
C. Morphine
EEG
EMG
D. Buprenorphine
EEG
EMG
200 µV
100 µV
5 s

Supplemental Table S 2.1. Descriptive statistics for Fig. 2.2.
Table values describe effects of treatment (saline, fentanyl, morphine, and buprenorphine) for 4 h (n = 10 mice).

Dependent Measure (corresponding fig.)	Treatment	Mean	Median	Minimum	Maximum	SD	%CV
%Wake (Fig. 2.2A)	Saline	37.6	38.8	27.5	48.0	7.4	19.7
	Fentanyl	81.6	83.8	56.4	97.4	12.7	15.6
	Morphine	97.1	96.8	94.7	100.0	2.3	2.4
	Buprenorphine	100.0	100.0	100.0	100.0	0.0	0.0
%NREM (Fig. 2.2B)	Saline	56.1	56.0	47.0	65.0	6.6	11.8
	Fentanyl	17.7	15.3	2.6	40.3	11.8	66.6
	Morphine	2.9	3.2	0.0	5.3	2.3	80.3
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A
%REM (Fig. 2.2C)	Saline	6.3	6.2	4.2	8.1	1.2	19.3
	Fentanyl	0.7	0.3	0.0	3.2	1.0	142.9
	Morphine	0.0	0.0	0.0	0.0	0.0	N/A
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A
# Wake Episodes (Fig. 2.2D)	Saline	35.4	38.5	16.0	46.0	10.5	29.6
	Fentanyl	14.9	14.5	6.0	32.7	8.2	54.8
	Morphine	5.8	4.7	1.0	12.0	4.5	78.8
	Buprenorphine	1.0	1.0	1.0	1.0	0.0	0.0
# NREM Episodes (Fig. 2.2E)	Saline	36.2	37.5	17.0	46.0	10.1	28.0
	Fentanyl	15.7	15.4	6.0	32.7	8.0	50.9
	Morphine	5.9	6.7	0.0	11.3	4.4	74.8
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A
# REM Episodes (Fig. 2.2F)	Saline	12.4	12.5	7.0	18.0	3.4	27.0
	Fentanyl	1.3	0.5	0.0	6.0	1.9	148.8
	Morphine	0.0	0.0	0.0	0.0	0.0	N/A
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A
Duration (min) of Wake Episodes (Fig. 2.2G)	Saline	2.5	2.4	1.7	3.4	0.6	24.9
	Fentanyl	28.2	25.3	2.5	84.6	23.8	84.2
	Morphine	115.4	85.5	18.9	240.0	90.9	78.7
	Buprenorphine	240.0	240.0	240.0	240.0	0.0	0.0
Duration (min) of NREM episodes (Fig. 2.2H)	Saline	4.1	3.7	2.6	6.6	1.4	33.7
	Fentanyl	15.7	15.4	6.0	32.7	8.0	50.9
	Morphine	5.9	6.7	0.0	11.3	4.4	74.8
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A
Duration (min) of REM Episodes (Fig. 2.4I)	Saline	1.3	1.3	0.8	1.6	0.2	18.4
	Fentanyl	0.5	0.2	0.0	1.5	0.6	123.1
	Morphine	0.0	0.0	0.0	0.0	0.0	N/A
	Buprenorphine	0.0	0.0	0.0	0.0	0.0	N/A

Supplemental Table S 2.2. Inferential statistics and effect size for Fig. 2.2.
Results from one-way, repeated measures ANOVA are reported for effects of treatment (saline, fentanyl, morphine, and buprenorphine) and mouse (n = 10) for 4 h.

Dependent Measure (corresponding fig.)	**ANOVA Table**	$\mathbf{F_{DFn, DFd}}$	***P***	**Sum of Squares**	**Eta Squared**
%Wake (Fig. 2.2A)	Treatment main effect	$F_{3, 27} = 165.9$	< 0.0001	24898	0.92599
	Mouse main effect	$F_{9, 27} = 1.419$	= 0.2292	638.9	0.02376
	Residual			1351	0.05025
%NREM (Fig. 2.2B)	Treatment main effect	$F_{3, 27} = 156.5$	< 0.0001	20015	0.92205
	Mouse main effect	$F_{9, 27} = 1.409$	= 0.2332	540.5	0.02490
	Residual			1151	0.05302
%REM (Fig. 2.2C)	Treatment main effect	$F_{3, 27} = 139.3$	< 0.0001	275.4	0.92323
	Mouse main effect	$F_{9, 27} = 0.865$	= 0.5664	5.131	0.01720
	Residual			17.79	0.05964
# Wake Episodes (Fig. 2.2D)	Treatment main effect	$F_{3, 27} = 60.23$	< 0.0001	6966	0.79730
	Mouse main effect	$F_{9, 27} = 2.105$	= 0.0654	730.2	0.08358
	Residual			1041	0.11915
# NREM Episodes (Fig. 2.2E)	Treatment main effect	$F_{3, 27} = 59.82$	< 0.0001	7559	0.81852
	Mouse main effect	$F_{9, 27} = 1.421$	= 0.2284	538.6	0.05832
	Residual			1137	0.12312
# REM Episodes (Fig. 2.2F)	Treatment main effect	$F_{3, 27} = 91.90$	< 0.0001	1091	0.88989
	Mouse main effect	$F_{9, 27} = 0.800$	= 0.6195	28.49	0.02324
	Residual			106.8	0.08711
Duration (min) of Wake Episodes (Fig. 2.2G)	Treatment main effect	$F_{3, 27} = 52.04$	< 0.0001	344565	0.81267
	Mouse main effect	$F_{9, 27} = 0.999$	= 0.4644	19838	0.04679
	Residual			59586	0.14054
Duration (min) of NREM Episodes (Fig. 2.2H)	Treatment main effect	$F_{3, 27} = 26.65$	< 0.0001	97.11	0.67344
	Mouse main effect	$F_{9, 27} = 1.311$	= 0.2772	14.32	0.09931
	Residual			32.79	0.22739

Supplemental Table S 2.2 Continued

Dependent Measure (corresponding fig.)	Dependent Measure (corresponding fig.)	Dependent Measure (corresponding fig.)	Dependent Measure (corresponding fig.)	Dependent Measure (corresponding fig.)	Dependent Measure (corresponding fig.)	ANOVA Table	ANOVA Table	ANOVA Table	ANOVA Table	ANOVA Table	ANOVA Table
Duration (min) of REM Episodes (Fig. 2.2I)	Treatment main effect	$F_{3, 27} = 34.67$	< 0.0001	11.27	0.76667						
	Mouse main effect	$F_{9, 27} = 0.521$	= 0.8461	0.5082	0.03457						
	Residual			2.924	0.19891						

Supplemental Table S 2.3. Descriptive statistics for Fig. 2.4.
Table values describe effects of treatment (saline, fentanyl, morphine, and buprenorphine) across one light/dark cycle (n = 6 mice).

		Light Phase					
Dependent Measure (corresponding figure)	**Treatment**	**Mean**	**Median**	**Min**	**Max**	**SD**	**%CV**
%Wake (Fig. 2.4A)	Saline	34.5	34.1	31.8	37.1	1.9	5.6
	Fentanyl	58.7	60.3	42.8	68.1	9.5	16.2
	Morphine	49.5	52.3	40.3	54.8	6.0	12.1
	Buprenorphine	49.5	52.3	40.3	54.8	6.0	12.1
%NREM (Fig. 2.4B)	Saline	59.3	59.3	56.6	61.2	1.7	2.8
	Fentanyl	38.0	36.3	28.9	53.2	9.0	23.7
	Morphine	45.8	44.1	40.1	54.2	5.3	11.6
	Buprenorphine	7.2	5.9	0.6	19.2	6.7	92.9
%REM (Fig. 2.4C)	Saline	6.2	6.4	4.5	7.1	0.9	15.3
	Fentanyl	3.1	3.3	2.1	3.9	0.7	24.1
	Morphine	4.7	4.7	3.0	6.3	1.2	24.7
	Buprenorphine	0.1	0.0	0.0	0.3	0.1	244.9
# Wake Episodes (Fig. 2.4D)	Saline	113.3	115.0	84.0	130.0	16.3	14.4
	Fentanyl	55.2	54.5	38.0	68.0	11.4	20.7
	Morphine	70.3	60.5	51.0	115.0	23.5	33.4
	Buprenorphine	26.3	27.0	8.0	48.0	14.6	55.5
# NREM Episodes (Fig. 2.4E)	Saline	114.2	116.5	85.0	130.0	16.5	14.4
	Fentanyl	53.8	50.5	38.0	68.0	11.6	21.5
	Morphine	70.7	60.5	52.0	116.0	23.6	33.3
	Buprenorphine	26.3	27.5	7.0	48.0	14.8	56.3
# REM Episodes (Fig. 2.4F)	Saline	35.2	35.0	28.0	42.0	5.0	14.2
	Fentanyl	13.5	12.5	8.0	20.0	4.3	32.0
	Morphine	22.0	19.0	14.0	33.0	7.7	34.9
	Buprenorphine	0.5	0.0	0.0	3.0	1.2	244.9
Duration (min) Wake Episodes (Fig. 2.4G)	Saline	1.7	1.7	1.5	1.9	0.2	8.8
	Fentanyl	5.6	6.1	2.2	9.1	3.0	52.6
	Morphine	4.1	4.4	2.2	5.7	1.6	39.7
	Buprenorphine	26.0	16.2	12.0	77.7	25.5	98.0
Duration (min) NREM Episodes (Fig. 2.4H)	Saline	3.3	3.1	2.8	4.4	0.6	18.7
	Fentanyl	53.8	50.5	38.0	68.0	11.6	21.5
	Morphine	70.7	60.5	52.0	116.0	23.6	33.3
	Buprenorphine	26.3	27.5	7.0	48.0	14.8	56.3
Duration (min) REM Episodes (Fig. 2.4I)	Saline	1.1	1.1	1.0	1.2	0.1	5.4
	Fentanyl	1.5	1.4	1.2	1.8	0.2	15.4
	Morphine	1.4	1.4	1.0	1.6	0.2	14.9
	Buprenorphine	0.1	0.0	0.0	0.7	0.3	244.9

Supplemental Table S 2.3. Continued

		Dark Phase					
Dependent Measure (corresponding figure)	**Treatment**	**Mean**	**Median**	**Min**	**Max**	**SD**	**%CV**
%Wake (Fig. 2.4A)	Saline	58.0	57.8	51.5	65.6	5.6	9.6
	Fentanyl	43.4	45.0	35.1	49.9	5.5	12.6
	Morphine	44.7	43.3	38.3	53.5	6.3	14.0
	Buprenorphine	44.7	43.3	38.3	53.5	6.3	14.0
%NREM (Fig. 2.4B)	Saline	39.7	39.3	33.1	45.9	4.9	12.4
	Fentanyl	51.2	50.2	44.8	58.7	5.1	9.9
	Morphine	50.6	51.9	42.6	56.9	6.0	11.9
	Buprenorphine	55.5	55.9	47.2	60.2	4.8	8.6
%REM (Fig. 2.4C)	Saline	2.4	2.3	1.3	4.1	1.1	44.3
	Fentanyl	5.4	5.4	4.2	6.2	0.7	12.2
	Morphine	4.4	4.2	3.9	5.7	0.7	16.0
	Buprenorphine	5.9	5.8	4.8	6.9	0.7	12.4
# Wake Episodes (Fig. 2.4D)	Saline	125.7	134.5	81.0	169.0	33.2	26.4
	Fentanyl	123.0	124.0	105.0	138.0	10.7	8.7
	Morphine	130.5	128.0	116.0	146.0	12.7	9.7
	Buprenorphine	135.3	130.5	88.0	199.0	40.9	30.3
# NREM Episodes (Fig. 2.4E)	Saline	134.2	137.5	92.0	168.0	24.7	18.4
	Fentanyl	122.3	123.5	104.0	138.0	11.0	9.0
	Morphine	129.3	127.5	115.0	145.0	13.5	10.4
	Buprenorphine	134.8	130.0	86.0	198.0	41.9	31.1
# REM Episodes (Fig. 2.4F)	Saline	19.3	19.0	9.0	29.0	8.3	43.1
	Fentanyl	32.3	32.0	26.0	42.0	6.2	19.0
	Morphine	29.2	28.0	24.0	39.0	5.6	19.1
	Buprenorphine	36.0	36.0	30.0	40.0	3.6	9.9
Duration (min) Wake Episodes (Fig. 2.4G)	Saline	3.2	3.1	2.2	5.1	1.0	31.4
	Fentanyl	2.5	2.5	2.0	3.1	0.4	15.4
	Morphine	2.5	2.5	1.9	3.1	0.5	20.0
	Buprenorphine	2.0	1.9	1.2	2.9	0.6	29.4
Duration (min) NREM Episodes (Fig. 2.4H)	Saline	2.1	2.1	1.9	2.5	0.2	10.3
	Fentanyl	3.0	3.1	2.5	3.4	0.4	13.6
	Morphine	2.8	2.8	2.3	3.3	0.3	12.0
	Buprenorphine	3.1	3.2	2.2	4.0	0.7	23.2
Duration (min) REM Episodes (Fig. 2.4I)	Saline	0.9	0.9	0.7	1.3	0.3	28.6
	Fentanyl	1.1	1.2	0.5	1.5	0.3	30.3
	Morphine	1.1	1.1	0.9	1.2	0.1	10.0
	Buprenorphine	1.2	1.2	1.0	1.4	0.1	12.1

Supplemental Table S 2.4. Inferential statistics and effect size for Fig. 2.4.
Results from two-way, repeated measures ANOVA are reported for effects of treatment (saline, fentanyl, morphine, and buprenorphine), phase (light or dark), and treatment by phase interaction (n = 6 mice).

Dependent Measure (corresponding fig.)	**ANOVA Table**	**$F_{DFn, DFd}$**	***P***	**Sum of Squares**	**Eta Squared**
%Wake (Fig. 2.4A)	Treatment main effect	$F_{3, 30} = 23.21$	< 0.0001	2960	0.19173
	Phase main effect	$F_{1, 10} = 89.60$	< 0.0001	1928	0.12489
	Treatment x phase interaction	$F_{3, 30} = 72.74$	< 0.0001	9275	0.60079
	Residual			1275	0.08259
%NREM (Fig. 2.4B)	Treatment main effect	$F_{3, 30} = 22.73$	< 0.0001	2482	0.18882
	Phase main effect	$F_{1, 10} = 70.69$	< 0.0001	1645	0.12514
	Treatment x phase interaction	$F_{3, 30} = 65.14$	< 0.0001	7116	0.54135
	Residual			1092	0.14469
%REM (Fig. 2.4C)	Treatment main effect	$F_{3, 30} = 10.93$	< 0.0001	18.17	0.09328
	Phase main effect	$F_{1, 10} = 11.25$	= 0.0073	11.60	0.05955
	Treatment x phase interaction	$F_{3, 30} = 89.30$	= 0.0001	148.4	0.76185
	Residual			16.62	0.08532
# Wake Episodes (Fig. 2.4D)	Treatment main effect	$F_{3, 30} = 5.767$	= 0.0031	10093	0.11423
	Phase main effect	$F_{1, 10} = 130.8$	< 0.0001	46625	0.52769
	Treatment x phase interaction	$F_{3, 30} = 8.079$	= 0.0004	14138	0.16001
	Residual			17501	0.19807
# NREM Episodes (Fig. 2.4E)	Treatment main effect	$F_{3, 30} = 8.808$	= 0.0002	13082	0.14723
	Phase main effect	$F_{1, 10} = 111.6$	< 0.0001	49024	0.55174
	Treatment x phase interaction	$F_{3, 30} = 8.808$	= 0.0005	11895	0.13387
	Residual			14853	0.16716
# REM Episodes (Fig. 2.4F)	Treatment main effect	$F_{3, 30} = 5.809$	< 0.0001	555.7	0.07841
	Phase main effect	$F_{1, 10} = 49.06$	< 0.0001	1564	0.22068
	Treatment x phase interaction	$F_{3, 30} = 43.77$	= 0.0022	4187	0.59079
	Residual			780.4	0.11012

Supplemental Table S 2.4. Continued

Dependent Measure (corresponding fig.)	ANOVA Table	$F_{DFn, DFd}$	*P*	Sum of Squares	Eta Squared
Duration (min) of Wake Episodes (Fig. 2.4G)	Treatment main effect	$F_{3, 30} = 3.949$	= 0.0174	1043	0.19111
	Phase main effect	$F_{1, 10} = 8.370$	= 0.0160	554.7	0.10164
	Treatment x phase interaction	$F_{3, 30} = 4.610$	= 0.0091	1218	0.22317
	Residual			2642	0.48409
Duration (min) of NREM Episodes (Fig. 2.4H)	Treatment main effect	$F_{3, 30} = 16.58$	< 0.0001	15.71	0.32416
	Phase main effect	$F_{1, 10} = 3.443$	= 0.0932	4.284	0.08840
	Treatment x phase interaction	$F_{3, 30} = 20.03$	< 0.0001	18.99	0.39184
	Residual			9.480	0.19561
Duration (min) of REM Episodes (Fig. 2.4I)	Treatment main effect	$F_{3, 30} = 27.05$	< 0.0001	3.031	0.36364
	Phase main effect	$F_{1, 10} = 0.509$	= 0.4918	0.04006	0.00481
	Treatment x phase interaction	$F_{3, 30} = 36.99$	< 0.0001	4.144	0.49718
	Residual			1.120	0.13437

Supplemental Table S 2.5. Descriptive statistics for Figs. 2.5*A-C*.
Table values describe effects of treatment (saline, fentanyl, morphine, and buprenorphine) for 24 h (n= 6 mice).

Dependent Measure (corresponding figure)	**Time (h)**	**Treatment**							
		Saline		**Fentanyl**		**Morphine**		**Buprenorphine**	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
% Wake (Fig. 2.5A)	1	73.33	37.43	100.00	0.00	98.52	3.63	100.00	0.00
	2	29.49	24.84	98.56	3.52	96.25	9.19	100.00	0.00
	3	32.41	17.04	92.69	17.92	87.45	30.73	100.00	0.00
	4	38.43	16.22	83.33	35.90	68.10	41.47	100.00	0.00
	5	22.64	28.41	62.59	37.61	38.52	11.07	100.00	0.00
	6	32.31	23.76	43.47	29.81	13.24	8.93	100.00	0.00
	7	28.89	20.17	31.20	19.43	26.11	17.22	97.96	4.99
	8	51.99	12.11	20.51	8.82	24.63	9.69	88.56	20.46
	9	33.10	19.48	31.11	12.04	18.61	14.95	77.73	26.02
	10	44.91	17.01	30.51	16.52	25.60	8.10	76.20	25.05
	11	51.67	13.63	38.61	14.85	47.36	16.23	66.71	25.66
	12	61.85	34.14	61.44	16.14	51.71	13.06	67.78	21.83
	13	44.35	22.36	27.27	8.38	40.74	12.61	57.82	25.44
	14	53.19	11.93	36.16	11.10	35.83	11.77	37.50	13.53
	15	58.47	31.41	46.02	17.66	46.30	13.08	33.94	18.23
	16	53.75	27.94	48.33	26.10	37.13	22.61	25.32	9.66
	17	43.84	21.61	54.07	23.95	39.17	6.86	20.93	9.59
	18	42.31	30.17	39.49	13.04	37.92	11.86	27.27	7.98
	19	35.32	15.49	26.90	14.66	30.97	11.72	24.81	12.37
	20	43.33	9.29	29.58	7.61	37.73	14.68	21.30	14.56
	21	54.68	20.51	39.35	12.38	50.23	13.15	34.72	13.28
	22	74.49	12.74	64.81	26.05	62.22	17.61	53.19	22.37
	23	51.16	15.85	66.11	14.70	71.94	27.10	44.03	21.07
	24	32.18	12.14	37.36	19.44	42.73	27.07	53.70	33.81
% NREM (Fig. 2.5B)	1	9.72	11.25	0.00	0.00	1.48	3.63	0.00	0.00
	2	75.00	4.57	1.44	3.52	3.75	9.19	0.00	0.00
	3	64.91	7.96	7.31	17.92	11.67	28.58	0.00	0.00
	4	55.09	14.90	15.65	33.43	29.58	38.01	0.00	0.00
	5	79.91	3.80	36.30	36.65	57.92	10.40	0.00	0.00
	6	69.86	11.48	51.81	26.16	78.15	8.91	0.00	0.00
	7	70.00	6.10	64.58	18.79	63.94	14.61	2.04	4.99
	8	49.35	13.43	71.02	8.72	68.19	7.95	11.44	20.46
	9	64.58	8.80	62.22	10.28	71.71	12.99	22.27	26.02
	10	57.36	11.91	63.01	14.56	67.55	7.45	23.84	24.65
	11	40.69	10.16	55.09	10.91	47.96	16.44	31.85	22.73
	12	21.16	18.31	36.48	15.47	44.63	11.12	30.46	19.70

Supplemental Table S 2.5. Continued

Dependent Measure (corresponding figure)	Time (h)	Treatment							
		Saline		Fentanyl		Morphine		Buprenorphine	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
% NREM (Fig. 2.5B)	13	40.23	19.78	65.28	7.36	55.37	11.07	39.40	22.75
	14	40.14	6.71	55.88	9.31	57.36	9.34	58.24	12.91
	15	29.21	22.86	47.92	13.99	48.24	12.41	58.29	14.82
	16	42.87	26.35	46.81	22.09	57.41	20.36	65.19	7.51
	17	39.12	18.93	42.96	22.60	55.56	5.47	70.14	9.85
	18	51.39	26.98	54.35	10.64	55.93	11.05	66.16	7.02
	19	61.48	13.12	64.95	12.62	62.18	10.75	65.88	11.59
	20	54.68	5.92	64.81	8.14	55.56	14.00	70.88	11.98
	21	35.42	13.43	56.30	10.30	48.01	11.36	58.61	11.28
	22	26.57	16.47	33.33	23.76	36.57	16.72	43.15	20.22
	23	43.38	13.40	32.41	14.54	26.81	25.69	53.66	19.90
	24	51.34	19.66	58.70	16.83	47.04	27.54	43.06	31.77
% REM (Fig. 2.5C)	1	0.28	0.68	0.00	0.00	0.00	0.00	0.00	0.00
	2	6.53	1.11	0.00	0.00	0.00	0.00	0.00	0.00
	3	8.47	0.97	0.00	0.00	0.88	2.15	0.00	0.00
	4	5.79	2.53	1.02	2.49	2.31	3.65	0.00	0.00
	5	9.07	1.65	0.97	2.25	3.43	3.56	0.00	0.00
	6	6.16	2.21	4.12	4.73	6.62	3.16	0.00	0.00
	7	7.82	2.19	3.84	2.58	9.95	4.13	0.00	0.00
	8	6.06	1.31	8.33	3.37	6.99	2.80	0.00	0.00
	9	6.67	3.58	6.57	2.45	9.68	3.51	0.00	0.00
	10	5.56	2.22	6.76	2.87	7.13	2.43	0.23	0.57
	11	2.73	2.02	6.30	4.26	4.63	1.17	1.44	3.52
	12	0.32	0.79	2.08	1.75	3.29	1.64	1.71	2.69
	13	1.62	2.20	7.45	2.26	3.89	2.18	2.45	3.64
	14	1.67	2.11	7.96	2.23	6.81	2.83	4.26	3.94
	15	2.59	4.31	6.06	4.00	5.46	2.77	7.78	3.75
	16	1.34	1.39	4.86	4.51	5.46	2.92	9.49	2.33
	17	3.06	2.98	2.96	3.24	5.19	1.88	8.94	1.73
	18	4.95	4.41	6.16	2.54	5.51	1.59	6.57	2.71
	19	5.32	1.91	8.15	3.71	6.57	1.53	9.31	2.27
	20	3.19	2.53	5.60	1.40	4.68	2.13	7.82	3.58
	21	0.56	1.36	4.35	2.75	1.76	2.40	6.57	3.14
	22	1.30	1.32	1.85	2.48	1.20	1.90	3.66	2.95
	23	2.78	2.43	1.48	0.89	1.25	1.45	2.31	1.66
	24	4.26	3.34	3.89	2.94	3.24	3.30	3.19	2.59

Supplemental Table S 2.6. Inferential statistics and effect size for Fig. 2.5.
Results from two-way, repeated measures ANOVA (Figs. 2.5*A – C*) are reported for effects of treatment (saline, fentanyl, morphine, and buprenorphine), time (24 h of recording), and treatment by time interaction (n = 6 mice). Results from one-way, repeated measures ANOVA (Fig. 2.5*D* and *E*) are reported for treatment effects on latency to onset of NREM sleep and REM sleep (n = 6 mice).

Dependent Measure (corresponding fig.)	**ANOVA Table**	**$F_{DFn, DFd}$**	***P***	**Sum of Squares**	**Eta Squared**
%Wake (Fig. 5A)	Treatment main effect	$F_{3, 15} = 40.11$	< 0.0001	27200	0.05396
	Time main effect	$F_{23, 115} = 19.30$	< 0.0001	143125	0.28395
	Treatment x time interaction	$F_{69, 345} = 5.956$	< 0.0001	158774	0.31500
	Subject			1189	0.00236
	Residual			133295	0.26445
%NREM (Fig. 5B)	Treatment main effect	$F_{3, 15} = 24.49$	< 0.0001	20621	0.05038
	Time main effect	$F_{23, 115} = 19.03$	< 0.0001	117341	0.28670
	Treatment x time interaction	$F_{69, 345} = 7.995$	< 0.0001	144762	0.35370
	Subject			990.4	0.00242
	Residual			90529	0.22119
%REM (Fig. 5C)	Treatment main effect	$F_{3, 15} = 12.88$	= 0.0002	133.3	0.01678
	Time main effect	$F_{23, 115} = 9.242$	< 0.0001	1697	0.21362
	Treatment x time interaction	$F_{69, 345} = 8.018$	< 0.0001	3111	0.39161
	Subject			93.16	0.01173
	Residual			1940	0.24421
NREM Latency (min) (Fig. 5D)	Treatment main effect	$F_{3, 15} = 38.50$	< 0.0001	160.9	0.87020
	Mouse main effect	$F_{5, 15} = 0.4349$	= 0.8173	3.03	0.01639
	Residual			20.9	0.11303
REM Latency (min) (Fig. 5E)	Treatment main effect	$F_{3, 15} = 59.10$	< 0.0001	405.8	0.90925
	Mouse main effect	$F_{5, 15} = 0.5320$	= 0.7489	6.089	0.01364
	Residual			34.34	0.07694

Supplemental Table S 2.7. Descriptive statistics for Fig. 2.6.
Table values describe effects of treatment (saline, fentanyl, morphine, and buprenorphine) on EEG power (dB) during the first 30 min after injection, when all mice (n = 10) were awake.

Dependent Measure	Treatment	Mean	Median	Minimum	Maximum	SD	%CV
Total Power (0 – 60 Hz)	Saline	1095.6	1138.7	731.8	1415.5	234.0	21.4
	Fentanyl	945.2	977.3	412.4	1451.3	359.3	38.0
	Morphine	953.6	991.8	398.4	1341.5	279.3	29.3
	Buprenorphine	1075.1	1073.3	701.6	1405.2	229.3	21.3
Delta (0.5 – 4 Hz)	Saline	143.6	150.1	107.9	184.8	23.1	16.1
	Fentanyl	134.8	135.2	98.7	168.4	22.4	16.6
	Morphine	132.1	135.6	95.1	158.9	22.5	17.0
	Buprenorphine	137.8	137.5	109.8	167.6	19.1	13.9
Theta (4 – 8 Hz)	Saline	147.6	156.7	109.5	170.9	20.5	13.9
	Fentanyl	123.2	115.6	76.1	168.5	32.7	26.5
	Morphine	128.3	136.8	82.7	152.3	23.4	18.2
	Buprenorphine	139.9	140.2	102.6	172.2	22.3	16.0
Alpha (8 – 13 Hz)	Saline	144.7	150.3	107.5	174.3	23.7	16.4
	Fentanyl	107.4	105.4	40.4	166.5	45.2	42.1
	Morphine	112.2	118.0	45.5	156.0	30.8	27.4
	Buprenorphine	133.5	141.2	97.7	163.5	26.1	19.5
Sigma (12 – 15 Hz)	Saline	64.7	66.4	46.5	85.7	12.5	19.3
	Fentanyl	49.4	49.7	13.7	83.2	22.9	46.4
	Morphine	49.7	53.3	17.9	67.7	15.2	30.6
	Buprenorphine	58.5	58.2	40.1	75.0	11.9	20.3
Beta (13-30 Hz)	Saline	278.8	275.6	168.9	397.2	74.2	26.6
	Fentanyl	232.2	256.5	63.2	412.1	110.9	47.7
	Morphine	231.9	229.2	71.4	357.6	84.9	36.6
	Buprenorphine	262.8	255.5	161.8	373.1	69.2	26.3
Gamma 30 – 60 Hz)	Saline	144.7	150.3	107.5	174.3	23.7	16.4
	Fentanyl	107.4	105.4	40.4	166.5	45.2	42.1
	Morphine	112.2	118.0	45.5	156.0	30.8	27.4
	Buprenorphine	133.5	141.2	97.7	163.5	26.1	19.5

Supplemental Table S 2.8. Inferential statistics and effect size for Fig. 2.6.
Results from one-way, repeated measures ANOVA are reported for effects of treatment (saline, fentanyl, morphine, and buprenorphine) and mouse (n = 10) on EEG power (percent change from saline).

Dependent Measure	**ANOVA Table**	$\mathbf{F_{DFn, DFd}}$	***P***	**Sum of Squares**	**Eta Squared**
Total Power (0 – 60 Hz)	Treatment main effect	$F_{3, 27} = 9.550$	= 0.0002	6178	0.27370
	Mouse main effect	$F_{9, 27} = 5.448$	= 0.0003	10572	0.46837
	Residual			5822	0.25793
Delta (0.5 – 4 Hz)	Treatment main effect	$F_{3, 27} = 0.6231$	= 0.6062	301.7	0.04464
	Mouse main effect	$F_{9, 27} = 1.445$	= 0.2188	2098	0.31045
	Residual			4358	0.64487
Theta (4 – 8 Hz)	Treatment main effect	$F_{3, 27} = 4.668$	= 0.0094	1796	0.22743
	Mouse main effect	$F_{9, 27} = 2.288$	= 0.0467	2640	0.33430
	Residual			3462	0.43839
Alpha (8 – 13 Hz)	Treatment main effect	$F_{3, 27} = 7.198$	= 0.0011	5032	0.28405
	Mouse main effect	$F_{9, 27} = 3.048$	= 0.0119	6392	0.36082
	Residual			6292	0.35518
Sigma (12 – 15 Hz)	Treatment main effect	$F_{3, 27} = 6.527$	= 0.0018	4724	0.24999
	Mouse main effect	$F_{9, 27} = 3.527$	= 0.0053	7658	0.40525
	Residual			6514	0.34471
Beta (13-30 Hz)	Treatment main effect	$F_{3, 27} = 2.365$	= 0.0932	2667	0.11677
	Mouse main effect	$F_{9, 27} = 2.962$	= 0.0139	10023	0.43884
	Residual			10150	0.44440
Gamma (30 – 60 Hz)	Treatment main effect	$F_{3, 27} = 0.7923$	= 0.5088	2453	0.04488
	Mouse main effect	$F_{9, 27} = 2.621$	= 0. 0254	24345	0.44541
	Residual			27860	0.50972

Chapter 3 : Fentanyl and morphine dissociate relationships among states of sleep and wakefulness, EEG power, motor activity and body temperature in C57BL/6J mice

This chapter has been adapted for dissertation formatting from a manuscript to be submitted for publication in a peer-reviewed journal:

Fentanyl and morphine dissociate relationships among states of sleep and wakefulness, EEG power, motor activity, and body temperature in C57BL/6J mice

Zebadúa Unzaga D, O'Brien CB, Cooper WN, Olson CE, Zhu X, O'Neil MA, Price JM, Lydic R, Baghdoyan HA

My contributions to the publication presented and adapted in the following chapter include data collection, analysis, and interpretation. Additionally, I contributed to the writing of the manuscript and figure preparation.

Abstract

States of sleep and wakefulness, electroencephalographic (EEG) power, motor activity, and body temperature are temporally linked, yet these variables are often studied independently. This study obtained the foregoing measures simultaneously, making it possible to test the hypothesis that antinociceptive doses of fentanyl and morphine simultaneously alter sleep-wake states, EEG power, motor activity, and subcutaneous body temperature (Tsc). Twelve C57BL/6J mice were used to identify the lowest doses of fentanyl (0.1 mg/kg) and morphine (1 mg/kg) that caused antinociception. Additional mice (n=12) were implanted with telemeters to simultaneously record EEG, electromyogram, motor activity, and Tsc. The results show that fentanyl (0.1, 0.3, 1, 3 mg/kg) and morphine (1, 3, 10, 30 mg/kg) significantly ($P<0.05$) increased wakefulness and decreased NREM and REM sleep. Higher doses of fentanyl and morphine significantly and differentially altered EEG power during wakefulness. Fentanyl (0.1 to 3 mg/kg) and morphine (3 to 30 mg/kg) significantly increased motor activity. Tsc during wakefulness was significantly

decreased by fentanyl (1, 3 mg/kg). Morphine increased (3 mg/kg) and decreased (30 mg/kg) Tsc. Mediation analyses showed that the increase in wakefulness caused by fentanyl and morphine was partially mediated by motor activity, but not by changes in Tsc. Compared to humans, these results in mice reveal similarities (sleep disruption, altered EEG power, hyperthermia and hypothermia) and differences (increased motor activity) in opioid-induced phenotypes. The results of mediation analyses highlight the need for a systems biology approach to studies using murine models of pain, addiction, and opioid-induced disruption of sleep and autonomic physiology.

Introduction

Despite the ongoing crisis of addiction, opioids are a vital tool for managing acute (Dowell et al., 2022) and chronic (Dahlhamer et al., 2018) pain. Factors that complicate the clinical usefulness of opioids include respiratory depression (Ramirez et al., 2021), abuse potential (Volkow & McLellan, 2016), sleep disruption (Eacret et al., 2020; R. Lydic et al., 2022), and slowing of cortical electroencephalographic (EEG) activity (Cao & Javaheri, 2018; Eacret et al., 2020; Greenwald & Roehrs, 2005; R. Lydic et al., 2022). In humans reduced sleep causes neurobehavioral deficits (Yamazaki et al., 2021) and increases the risk of addiction relapse (Brower & Perron, 2010; López-Muciño et al., 2022; Roehrs et al., 2021). Sleep disruption delays wound healing in mice (McLain et al., 2018) and humans (Anderson & Jeter, 2023). Sleep loss also causes hyperalgesia in humans (Finan et al., 2013; Roehrs et al., 2006), rats (Muncey et al., 2010; Vanini et al., 2014), and mice (Dalanon et al., 2021). Hyperalgesia increases analgesic requirement (Fletcher & Martinez, 2014; Knutson, 2015), creating a vicious cycle (Haack et al., 2020) for pain management.

Mediation analysis, a specific application of structural equation modeling, is a statistical approach used to understand the mechanism through which an independent variable (predictor) influences a dependent variable (outcome) via one or more intermediary variables, known as mediators. This report presents results of a study designed to evaluate the hypothesis that antinociceptive doses of fentanyl and morphine disrupt sleep while causing changes in cortical EEG power, motor activity, and subcutaneous body temperature (Tsc). For the present study a direct effect indicates how the opioid treatment impacts any one of the dependent variables without influence of a mediator variable. An indirect effect quantifies how the opioid dose alters a dependent variable through a mediator variable. The total effect of fentanyl or morphine is represented by the sum of the direct and indirect effects. Mediation analyses that incorporate the complexity of opioids causing direct and indirect effects have provided insights relevant to opioid-induced respiratory depression (Orr et al., 2024) and to clinical management of pain (Whibley et al., 2019). The present study is a novel application of mediation analysis to quantify opioid-induced changes in the neurobehavioral function of C57/BL6J (B6) mice.

Single-dose studies have reported opioid-induced sleep disruption and EEG slowing in B6 mice (C. O. O'Brien et al., 2021). The importance of quantifying dose-dependent effects of fentanyl has been emphasized (Burgraff, 2024). This study compared the effects of eight fentanyl and eight morphine doses on simultaneously obtained measures of cortical EEG activity, motor activity, and subcutaneous body temperature (Tsc). Mediation analysis (Vuorre & Bolger, 2018) was used to quantify the extent to which opioid-induced sleep disruption is mediated by changes in motor activity and/or Tsc. The results illustrate the power of a systems biology approach (Lydic & Baghdoyan, 2021) for unmasking opioid-induced changes in neurobehavioral function

that are the emergent product of interacting regulatory systems. Preliminary data quantifying the dose-response effects of fentanyl and morphine on states of sleep and wakefulness, EEG power, motor activity, and Tsc in B6 mice have been presented (Cooper et al., 2023; C. B. O'Brien et al., 2021; Zebadúa Unzaga et al., 2021; Zebadúa Unzaga et al., 2022)..

Methods

Animals

All experiments involving animals were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee and were conducted in accordance with the ARRIVE guidelines (Percie du Sert et al., 2020). Adult, male B6 mice (Stock# 000664; n=24) were purchased from The Jackson Laboratory (Bar Harbor, ME). At the University of Tennessee Knoxville mice were housed on a 12 h:12 h light-dark schedule in a facility where temperature and humidity ranged from 20.6 to 24.5 °C and 30 to 70%, with 10 to 12 air changes every hour. Mice had ad libitum access to water and rodent chow (Teklad 8640, Envigo, Madison, WI). Igloos, nestlets, and paper cones were rotated in home cages to provide environmental enrichment. Before surgical implantation of telemeters, littermates were housed together with no more than five mice in each cage (19 x 29 x 13 cm). Cages, enrichment items, and bedding (Harlan Soft Cob Enrichment Bedding) were changed once per week. Mice were monitored daily by laboratory members and by sensors that provided the investigators with continuous 24/7 wireless receipt of temperature and humidity data. Mice were given at least one-week to adjust to the housing environment before starting nociceptive testing or surgical procedures.

Quantifying antinociceptive effects of fentanyl and morphine

The tail withdrawal latency test measured the time from tail immersion into a beaker of water

maintained at 47.8 to 50°C to mouse-initiated tail removal (Gårdmark et al., 1998; Schildhaus et al., 2014). Mean ± SD age of these mice (n=12) was 26 ± 5.2 weeks and mean ± SD body weight was 35 ± 4.9 g. One week prior to measuring tail withdrawal latency the mice were conditioned daily to being handled and placed in an acrylic restraint tube with their tails extending outside of the tube. For data collection the distal third of the tail was placed in the water bath and a timer was started. The timer was stopped when the mouse withdrew its tail from the water. Three tail withdrawal latency measures were obtained from each mouse. The three measures were separated by a 10-s interval of no stimulation. Baseline tail withdrawal latency was measured prior to subcutaneous injections (0.3 mL) of saline and each dose of fentanyl and morphine. Tail withdrawal latency was measured again 30 min after saline or opioid administration. To prevent tissue damage, the maximum amount of time that mouse tails were left in the warm water was 15 s, after which the experimenter removed the mouse tails from the water bath. Thus, the maximum possible value for tail withdrawal latency was 15 s (Glovak et al., 2017).

Implantation of subcutaneous telemeters for simultaneous recording of EEG, electromyogram (EMG), motor activity, and subcutaneous body temperature (Tsc)

Twelve additional B6 mice naïve to opioids were implanted with telemeters (HD-X02, Data Sciences International, DSI, New Brighton, MN). Telemeters were used to quantify effects of opioids on states of sleep and wakefulness, cortical EEG power, dorsal neck muscle EMG, motor activity, and Tsc from freely behaving mice in their home cages. Surgeries were performed under general anesthesia with 2% isoflurane (Henry Schein, Melville, NY) delivered in 100% oxygen at a flow rate of 1 L/min. At the time of surgery mean ± SD mouse age was 22 ± 3.2

weeks and mean ± SD mouse body weight was 33 ± 4.2 g. Isoflurane delivery via an agent-specific vaporizer was monitored by spectrophotometry (Cardiocap/5; Datex-Ohmeda Inc., Madison WI).

Mice were placed in a plexiglass induction chamber and loss of righting reflex was used as an index of loss of consciousness (Vanini et al., 2014). Mice were shaved over the skull and cranial half of the dorsum. Anesthetic depth was assessed based on breathing rate and lack of response to a foot pad pinch. Mice were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga CA; model 962) fitted with a mouse adaptor (model 921) and a mouse anesthesia mask (model 907). Ketoprofen (5 mg/kg) in saline (0.3 mL) was administered subcutaneously to provide post-operative analgesia and prevent dehydration. A subcutaneous radio-frequency identification chip (ID-100B 1.4, MICROCHIPID, Lake Zurich, IL) was implanted to provide unique alphanumeric identification for each mouse. Throughout surgery the eyes of the mouse were protected with ophthalmic ointment. Core body temperature (36–37 oC) was monitored via a rectal thermometer and maintained using hot water circulating through a leakproof pad (Gaymar TP400 T/Pump heat therapy system). The skin was prepared aseptically for surgery by alternating iodine solution and iodine scrub. Respiratory rate and body temperature were measured every 10 min until the end of the surgical procedure.

A midline skin incision was made beginning at the top of the skull and extending caudally between the scapulae. Bipolar EEG recording electrodes originating from the telemeter were connected to stainless steel screws (Antrin Miniature Specialties, Fallbrook, CA) implanted above the cortex. Burr holes for the screws were made at stereotaxic coordinates 1.0 mm anterior to Bregma and 1.0 mm lateral to the midline (negative lead), and 3.0 mm posterior to

Bregma and 3.0 mm lateral to the midline (positive lead) (Paxinos & Franklin, 2019). Two electrodes were placed in the cervical trapezius muscle in the dorsal region of the neck to record EMG. The telemeter was placed in a deep subcutaneous pocket in the right dorsal flank. The dorsal portion of the incision over the skull and neck was closed with non-absorbable sutures (Prolene 6-0) in a simple interrupted pattern. The caudal portion of the incision was closed with wound clips (Henry Schein, Melville NY). Mice recovered in a heated cage and were monitored continuously until they displayed normal righting and ambulation.

After surgery, mice were housed individually to prevent disruption of the implant by cage mates. Daily health examinations were performed by investigators to assess mouse well-being. Postoperative monitoring focused on wound healing and behavioral indices of health including normal locomotion, nest building, and normal gain of body weight. Mice recovered during a two-week postsurgical healing period that preceded data collection.

Experimental design

A within-subject, repeated measures design was used to reduce the number of animals needed (Piantadosi, 1997). Each mouse received subcutaneous injections (0.3 mL) of saline (vehicle control) and increasing half-log doses of fentanyl (0.001 to 3 mg/kg) and morphine (0.01 to 30 mg/kg). Injections into the same mouse were separated by three to four days. To reduce the potential for order bias, each mouse was randomly assigned to receive either fentanyl or morphine treatment first. After each mouse received saline and all doses of the first opioid, mice were given one week of no injections before starting the second opioid. A second saline injection was administered before the second treatment protocol was started. Results of the first and second saline treatment were compared to confirm that the dependent measures of

nociception, sleep-wake states, motor activity, and Tsc had not changed due to treatment or time. Mice were weighed and given health examinations before each experiment.

Recording and quantifying states of sleep and wakefulness, motor activity, and subcutaneous body temperature

Recordings of the dependent measures were obtained from mice in their home cages by placing each cage on a receiver that communicated with the data acquisition computer (DSI Matrix 2.0 MX2). Subcutaneous injections were given within 90 min of light onset and 4-h recordings began immediately after each injection. EEG and EMG signals were used to objectively quantify states of wakefulness, rapid eye movement (REM) sleep, and non-REM (NREM) sleep. Motor activity was measured by the telemeter as activity counts in arbitrary units (a.u.). The implanted telemeter also measured Tsc in degrees Celsius. EEG, EMG, motor activity, and Tsc were measured and recorded simultaneously.

Ponemah software (v6.33, DSI) was used to digitize EEG, EMG, motor activity, and Tsc signals at a sampling frequency of 500 Hz. Bandpass filters of 0.3 to 30 Hz and 10 to 100 Hz were applied to the EEG and EMG signals, respectively. A notch filter of 60 Hz was applied to both signals to reduce electrical noise. The digitized biopotentials were scored offline using NeuroScore™ (v3.3.1, DSI).

Behavioral states of sleep and wakefulness were assessed in 10-s bins by two independent scorers, one of whom was blinded to the treatment condition. Agreement between the two scorers was equal to or greater than 90%. Criteria used to score each 10-s bin as wakefulness, NREM sleep, or REM sleep have been previously described (Flint et al., 2010; C. O. O'Brien et al., 2021). Briefly, episodes of wakefulness were characterized by a low amplitude, fast

frequency EEG signal and a high amplitude EMG signal. Epochs of NREM sleep were distinguished by increased EEG delta activity (0.5 to 4 Hz) and reduced EMG amplitude. REM sleep was identified by increased EEG theta activity (4 to 8 Hz) and a further decrease in EMG amplitude that indicated skeletal muscle atonia. Motor activity and Tsc were analyzed using a MATLAB custom script that averaged activity counts across the 4-h recording period during wakefulness and averaged Tsc during wakefulness, NREM sleep, and REM sleep.

Analysis of EEG power during wakefulness

The EEG was analyzed using multitaper spectral estimation (C. O. O'Brien et al., 2021) to assess opioid effects in five power bands: delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-13 Hz), sigma (12-15 Hz), and beta (13-30 Hz). Multitapered spectrograms were produced in MATLAB using custom scripts (O'Brien et al., 2019). All mice were awake during the first 30 min after receiving an injection of saline or an opioid. Accordingly, the first 30 min of recording post-injection was used to analyze EEG power. This approach ensured that any measured changes in EEG power were caused by the opioid and not by a change in behavioral state from wakefulness to sleep.

Statistical analyses

Opioid dose was treated as the independent variable throughout the study. Dependent variables included tail withdrawal latency, EEG power, percent of recording time spent in states of wakefulness, NREM sleep, and REM sleep, number of episodes and episode duration for each behavioral state, time from injection onset to enter NREM sleep and REM sleep (latency), number of transitions between states, motor activity, and Tsc. These dependent measures were analyzed using Prism (v10.2.3, GraphPad, San Diego, CA) or Statistical Analysis System (SAS

V9.4 TS1M7, Cary, NC) for descriptive and inferential statistics. Normality of these data was evaluated using Shapiro-Wilk and Q-Q plots.

The dose-response effects of fentanyl and morphine were analyzed using the non-linear regression equation Y= B + (T-B)/(1+10^(LogED50-X)). Terms in this equation were defined as: "B", the lower limit of the dependent variable; "T", the highest limit for the dependent variable response; "X", the logarithm of the fentanyl or morphine dose; and "Y", the dependent variable. Nonlinear regression was used to calculate the half-maximal dose (ED50) for increasing tail withdrawal latency, wakefulness, and motor activity, and the half-maximal dose for inhibiting (ID50) NREM sleep and REM sleep. Repeated measures, one-way ANOVA and post hoc Dunnett's multiple comparisons test were used to identify doses of fentanyl and morphine that caused significant changes from control (saline) for all dependent measures. The effects of opioids on sleep latency were also evaluated by survival analysis using the Kaplan-Meier method (Prism v10.2.3).

A within-subject multilevel mediation regression model (Vuorre & Bolger, 2018) was performed using Stata (StataCorp. 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC). These analyses assessed the possible mediation effects of motor activity and Tsc on the relationship between opioid dose and percent of the 4-h recording time spent in wakefulness. Statistical assumptions were evaluated as follows for the mediation analyses. Normality of residuals was performed for all models using the Shapiro-Wilk test and Q-Q plots. Equality of variance and outliers were evaluated using Levene's test for equality of variances, boxplots, and studentized residual diagnostics. Heteroskedasticity was evaluated using residual vs fitted plots. Motor activity data underwent a natural log (Ln) transformation in order to meet

the statistical assumptions of the model. After motor activity was transformed, all statistical assumptions were met. All comparisons were considered statistically significant with a probability (P) value of < 0.05.

Results

The overarching goal of this study was to elucidate the dose-dependent effects of fentanyl and morphine on measures of nociception, EEG power across five distinct frequency bands, body temperature, and locomotor activity. The dose-dependent effects of opioids on each of the above dependent variables was quantified in relation to the temporal organization of sleep and wakefulness. To achieve the study goal, the initial experiments quantified sleep-dependent changes in body temperature (**Figure 3.1A**) and dose-dependent effects of fentanyl and morphine on nociception (**Figure 3.1B**). These results logically prefaced quantification of opioid-induced alterations in EEG power (**Figure 3.2**) and the temporal organization of sleep/wake states (**Figure 3.3 and Table. 3.1**). **Figure 3.4** summarizes the time course for the effects of fentanyl and morphine on wakefulness, NREM sleep, and REM sleep. Finally, **Figure 3.5** plots the results of mediation analyses used to determine whether the opioid-induced increase in wakefulness was mediated by motor activity or Tsc. The 10 subsections below are organized to concisely describe the opioid effects on individual dependent variables and their interactions. Quantifying these interactions among multiple dependent variables was made possible by their simultaneous measurement as a function of fentanyl and morphine dose.

Sleep-dependent changes in subcutaneous body temperature (Tsc)

This study concurred with the admonition that "temperature measures should be obtained during animal sleep recordings to better understand the relation of sleep to thermoregulation" (Siegel,

2022). **Figure 3.1A** plots state-dependent changes in Tsc in B6 mice after injection of saline. These plots confirm that body temperature across the sleep/wake cycle was measured reliably in freely moving mice using the HD-X02 telemeter (Sun et al., 2023). Repeated measures one-way ANOVA indicated that Tsc varied significantly across the sleep/wake cycle ($F_{(2, 22)} = 202.8$; $P < 0.0001$). Tukey's multiple comparisons test revealed that Tsc was significantly (*$P < 0.0001$) decreased below waking levels during NREM sleep and during REM sleep, and that Tsc during REM sleep was significantly lower than Tsc during NREM sleep (#$P = 0.0052$).

Dose-dependent antinociception

Figure 3.1B summarizes the dose-response relationships for fentanyl- and morphine-induced antinociception. Tail withdrawal latency data were evaluated by one-way repeated measures ANOVA. There was a significant ($P < 0.0001$) main effect of fentanyl dose ($F_{7, 77} = 190.4$) and morphine dose ($F_{7, 77} = 154.8$) on tail withdrawal latency. Dunnett's multiple comparisons tests revealed that tail withdrawal latency was significantly increased above control levels by the four highest doses of fentanyl (*$P < 0.01$), and by the four highest doses of morphine (*$P < 0.02$). The lowest antinociceptive doses for fentanyl and morphine were 0.1 and 1 mg/kg, respectively. ED50 values for fentanyl and morphine were 0.49 and 8.29, respectively, indicating that, as expected, fentanyl was more potent than morphine in providing antinociception. The **Figure 3.1B** results are directly relevant to subsequent figures showing that the lowest dose of fentanyl and morphine that produced antinociception also disrupted sleep. The lowest antinociceptive dose of fentanyl also increased motor activity.

Fentanyl and morphine differentially altered sleep and EEG power during wakefulness

Time course graphs, or hypnograms, from one representative mouse (**Figure 3.2A**) plot effects

on sleep and wakefulness of the lowest effective antinociceptive doses of fentanyl (0.1 mg/kg) and morphine (1 mg/kg), as well as the highest tested doses of fentanyl (3 mg/kg) and morphine (30 mg/kg). **Figure 3.2A** illustrates opioid-induced sleep disruption by the lowest antinociceptive doses of both opioids and shows that the highest tested doses of fentanyl and morphine eliminated sleep for the entire 4-h recording period.

EEG data from the same mouse used for **Figure 3.2A** are plotted as multitaper spectrograms in **Figure 3.2B**. Spectrograms transform EEG signals from the time domain into the time-frequency domain. This transformation allows visualization of changes in spectral power (colors) as a function of drug and dose. Those changes can be seen by comparing the multitapered spectrograms of each dose of opioid with the respective control (saline), as well as by comparing the high dose fentanyl spectrogram (3 mg/kg) with the high dose morphine spectrogram (30 mg/kg).

Figure 3.2C illustrates the dose-dependent effects of fentanyl and morphine on EEG power during wakefulness in B6 mice. These effects were analyzed by one-way repeated measures ANOVA. Fentanyl (**Figure 3.2C**, blue symbols) and morphine (**Figure 3.2C**, red symbols) differentially altered EEG power. There was a significant main effect of fentanyl on EEG delta power ($F_{8, 80} = 20.66$; $P < 0.0001$), theta power ($F_{8, 80} = 3.84$; $P = 0.0007$), alpha power ($F_{8, 80} = 6.86$; $P < 0.0001$), and beta power ($F_{8, 80} = 6.13$; $P < 0.0001$). Dunnett's multiple comparisons tests revealed that relative to control (0 mg/kg), fentanyl at 1 and 3 mg/kg significantly (*$P<0.0001$) increased EEG delta power; at 3 mg/kg, significantly (*$P = 0.02$) increased theta power; and at 3 mg/kg significantly increased beta power (*$P < 0.0001$). Fentanyl at 0.3 and 1 mg/kg significantly (*$P < 0.04$) decreased alpha power (**Figure 3.2C**, left column).

In contrast to fentanyl, morphine did not significantly change EEG delta power (**Figure 3.2C**, right column). Morphine had a significant main effect on EEG theta power ($F_{8, 88} = 4.21$; P = 0.0003), alpha power ($F_{8, 88} = 6.93$; P < 0.0001), and beta power ($F_{8, 88} = 3.80$; P = 0.0007). Dunnett's multiple comparisons test revealed that, unlike fentanyl, morphine (30 mg/kg) decreased both EEG theta power, (*P = 0.03) and beta power (*P = 0.04). Similar to fentanyl, morphine at 10 and 30 mg/kg decreased EEG alpha power (*P < 0.01) (**Figure 3.2C**, right column). Neither fentanyl nor morphine significantly altered EEG sigma power (data not shown).

Fentanyl and morphine reduced time in NREM and REM sleep

The same doses of both opioids that caused significant antinociception (i.e., increased tail withdrawal latency; **Figure 3.1B**) also caused significant sleep disruption. **Figure 3.3A** shows that the percent of the 4-h recording period spent in wakefulness was progressively increased by increases in opioid dose and was accompanied by concomitant decreases in NREM sleep and REM sleep (**Figure 3.3B & 3.3C**). For all 12 mice, sleep was eliminated for at least 4 h by the highest dose of fentanyl (3 mg/kg) and by the two highest doses of morphine (10 and 30 mg/kg). The ED_{50} values for increasing wakefulness were 0.18 for fentanyl and 2.43 for morphine. The ID_{50} values for decreasing NREM sleep were 0.19 for fentanyl and 2.56 for morphine. REM sleep ID_{50} values for fentanyl and morphine were 0.13 and 1.50, respectively. Averaged together, the foregoing ED_{50} and ID_{50} values indicate that fentanyl was approximately 13 times more potent than morphine for increasing wakefulness and decreasing sleep.

The **Figure 3.3A, 3.3B, and 3.3C** data were analyzed using one-way repeated measures ANOVA to evaluate significant dose effects of fentanyl and morphine. There was a significant

($P < 0.0001$) treatment main effect for fentanyl on percent of the 4-h recording period spent in wakefulness (**Figure 3.3A**, $F_{8, 88} = 192.3$), NREM sleep (**Figure 3.3B**, $F_{8, 88} = 184.1$), and REM sleep (**Figure 3.3C**, $F_{8, 88} = 124.0$). Dunnett's multiple comparisons test indicated that doses of fentanyl ≥ 0.1 mg/kg significantly (*$P < 0.001$) increased percent wakefulness and decreased percent NREM (*$P < 0.001$) and REM sleep (*$P < 0.0001$) relative to control (0 mg/kg).

There was also a significant ($P < 0.0001$) treatment main effect of morphine on percent of the 4-h recording period spent in wakefulness (**Figure 3.3A**, $F_{8, 88} = 182.0$), NREM sleep (**Figure 3.3B**, $F_{8, 88} = 180.5$), and REM sleep (**Figure 3.3C**, $F_{8, 88} = 88.41$). Dunnett's multiple comparisons test revealed that doses of morphine ≥ 1 mg/kg significantly increased percent time in wakefulness (*$P < 0.001$) and decreased percent time in NREM sleep (*$P < 0.001$) and REM sleep (*$P < 0.0001$) relative to control (0 mg/kg).

Fentanyl and morphine decreased the number of sleep and wakefulness episodes

Both opioids caused dose-dependent decreases in the number of episodes of wakefulness, NREM sleep, and REM sleep (**Figure 3.3D-F).** The ID_{50} values for fentanyl-induced decreases in the number of episodes were 0.17, 0.18, and 0.11 for wakefulness, NREM sleep, and REM sleep, respectively. ID_{50} values for morphine-induced decreases in the number of episodes of wakefulness, NREM sleep, and REM sleep were 2.86, 2.94, and 1.69, respectively. When averaged together, these ID_{50} values indicate that fentanyl was approximately 16 times more potent than morphine for decreasing the number of behavioral state episodes.

One-way repeated measures ANOVA and post hoc Dunnett's multiple comparisons test were used to identify doses of fentanyl and morphine that significantly changed the number of episodes for each behavioral state. There was a significant ($P < 0.0001$) treatment main effect

for fentanyl on the number of episodes of wakefulness (**Figure 3.3D**, $F_{8, 88} = 80.16$), NREM sleep (**Figure 3.3E**, $F_{8, 88} = 82.72$), and REM sleep (**Figure 3.3F**, $F_{8, 88} = 97.65$). Dunnett's multiple comparisons test showed that doses of fentanyl ≥ 0.1 mg/kg significantly (*P < 0.001) decreased the number of episodes of all three behavioral states.

Significant (P < 0.0001) treatment main effects were found for morphine on the number of episodes of wakefulness (**Figure 3.3D**, $F_{8, 88} = 44.63$), NREM sleep (**Figure 3.3E**, $F_{8, 88} = 46.29$), and REM sleep (**Figure 3.3F**, $F_{8, 88} = 63.83$). Dunnett's multiple comparisons test revealed that doses of morphine ≥ 3 mg/kg significantly (*P < 0.0001) decreased the number of episodes of wakefulness (**Figure 3.3D**) and NREM sleep (**Figure 3.3E**). The number of REM sleep episodes (**Figure 3.3F**) was significantly (*P < 0.0001) decreased by doses of morphine ≥ 1 mg/kg.

Fentanyl and morphine increased the duration of waking episodes

The dose-dependent decreases in the number of episodes of wakefulness, NREM sleep, and REM sleep described above were accompanied by concomitant increases in the duration of wakefulness episodes (**Figure 3.3G**), and corresponding decreases in NREM and REM sleep episode duration (**Figure 3.3 H and 3.3 I)**. The ED_{50} values for opioid-induced increases in duration of wakefulness episodes were 0.59 for fentanyl and 8.70 for morphine, respectively (**Figure 3.3G**). ID_{50} values for opioid-induced decreases in duration of NREM sleep episodes were 1.11 for fentanyl and 13.6 for morphine (**Figure 3.3H)**. ID_{50} values for opioid-induced decreases in REM sleep episode duration were 0.38 for fentanyl and 5.25 for morphine (**Figure 3.3I**). These ED_{50} and ID_{50} values, when averaged together, indicate that fentanyl was approximately 14 times more potent than morphine for effects on duration of episodes for all

three behavioral states.

One-way repeated measures ANOVA showed a treatment main effect for fentanyl on duration of episodes for wakefulness (**Figure 3.3G**, $F_{8, 88} = 85.28$, $P < 0.0001$), NREM sleep (**Figure 3.3H**, $F_{8, 88} = 17.67$, $P < 0.0001$), and REM sleep (**Figure 3.3I**, $F_{8, 88} = 37.38$, $P < 0.0001$). Dunnett's multiple comparisons test indicated that doses of fentanyl ≥ 0.3 mg/kg significantly (*$P < 0.0001$) increased episode duration of wakefulness and decreased REM sleep episode duration. NREM sleep episode duration was significantly (*$P < 0.0001$) decreased by the two highest doses of fentanyl, 1 and 3 mg/kg. There was a treatment main effect of morphine on duration of wakefulness episodes (**Figure 3.3G**, $F_{8, 88} = 287.1$, $P < 0.0001$), NREM sleep (**Figure 3.3H**, $F_{8, 88} = 34.18$, $P < 0.0001$), and REM sleep (**Figure 3.3I**, $F_{8, 88} = 30.88$, $P < 0.0001$). Dunnett's multiple comparisons test indicated that the 3, 10, and 30 mg/kg doses of morphine significantly increased the duration of waking episodes (*$P < 0.02$) and decreased the duration of REM sleep episodes (*$P < 0.02$). The 10 and 30 mg/kg doses of morphine decreased duration of NREM sleep episodes (*$P < 0.0001$). Wake duration was increased by morphine (3, 10, and 30 mg/kg), NREM sleep duration was decreased by morphine (10 and 30 mg/kg), and REM sleep duration was decreased by morphine (3, 10, and 30 mg/kg). In summary, both opioids disrupted sleep architecture by significantly increasing the duration of wakefulness episodes (**Figure 3.3G**), thereby decreasing the number of wakefulness episodes (**Figure 3.3D**) and decreasing the number and duration of sleep episodes (**Figures 3.3 E, 3.3 F, 3.3 H, and 3.3 I**).

Sleep latency was increased by antinociceptive doses of fentanyl and morphine

Survival curves (**Figure 3.3J-M**) plot the dose-dependent increases in latency to onset of NREM sleep and REM sleep caused by fentanyl and morphine. The probability of no NREM sleep and

no REM sleep increased with increasing doses of fentanyl (**Figure 3.3J and 3.3K**, respectively). The highest dose of fentanyl (3 mg/kg) increased NREM sleep latency to greater than 240 min for all 12 mice (**Figure 3.3J**). The two highest doses of morphine (10 and 30 mg/kg) increased NREM sleep and REM sleep latency to greater than 240 min for all 12 mice (**Figure 3.3L and 3.3M**, respectively).

Nonlinear regression analyses were applied to the dose-response data for latency to onset of NREM and REM sleep. ED_{50} values for the opioid-induced increases in NREM sleep latency were 0.18 for fentanyl and 2.95 for morphine. ED_{50} values for REM sleep latencies were 0.14 for fentanyl and 1.62 for morphine. These results indicate that fentanyl was approximately 14 times more potent than morphine for inhibiting the onset of both NREM and REM sleep.

One-way repeated measures ANOVA showed a treatment main effect for fentanyl on latency to onset of NREM sleep ($F_{8, 88} = 189.9$, $P < 0.0001$) and REM sleep ($F_{8, 88} = 181.6$, $P < 0.0001$). Dunnett's multiple comparisons test indicated that doses of fentanyl ≥ 0.1 mg/kg significantly (*$P < 0.0001$) increased latency to onset of NREM and REM sleep. NREM and REM sleep latencies were significantly increased by fentanyl (0.1, 0.3, 1, and 3 mg/kg). There was a treatment main effect for morphine on latency to onset of NREM sleep ($F_{8, 88} = 191.3$, $P < 0.0001$) and REM sleep ($F_{8, 88} = 203.4$, $P < 0.0001$). Dunnett's multiple comparisons test indicated that doses of morphine ≥ 1 mg/kg significantly increased NREM sleep latency (*$P < 0.01$) and REM sleep latency (*$P < 0.001$).

Fentanyl and morphine changed the number of transitions between states of sleep and wakefulness

Table 3.1. summarizes the effects of fentanyl and morphine on the number of state transitions.

Repeated measures ANOVA and Dunnett's multiple comparisons test showed that, relative to control (0 mg/kg), the number of all state transitions was significantly (*$P < 0.01$) decreased by the lowest antinociceptive dose of fentanyl (0.1 mg/kg). The lowest antinociceptive dose of morphine (1 mg/kg) significantly decreased transitions from NREM to REM sleep, and from NREM sleep to wakefulness. The two higher doses of fentanyl and morphine significantly decreased all state transitions.

Time course of dose-dependent opioid-induced sleep disruption

The **Figure 3.4** radar plots show the time course of sleep and wakefulness after injection of saline (black lines, 0 mg/kg), the lowest effective antinociceptive doses of fentanyl and morphine (blue lines and symbols, 0.1 and 1 mg/kg, respectively) and the next highest doses of fentanyl and morphine (green lines and symbols, 0.3 and 3 mg/kg, respectively). Red lines illustrate changes in behavioral state caused by the highest tested doses of fentanyl (3 mg/kg) and morphine (30 mg/kg). The 4-h recording period is divided into 30 min bins. Wakefulness did not return to control levels after administration of the two highest doses of fentanyl (**Figure 3.4A**) and morphine (**Figure 3.4D**). NREM sleep and REM sleep returned to control levels within 121-150 min after injection of the lowest antinociceptive doses of fentanyl (**Figure 3.4B**, 0.1 mg/kg) and morphine (**Figure 3.4E**, 1 mg/kg). NREM sleep and REM sleep did not return to control levels within the 240-min recording period after the 0.3 and 3 mg/kg doses of fentanyl (**C**) or after the 30 mg/kg dose of morphine (**Figure 3.4F**).

The **Figure 3.4** data were analyzed by two-way repeated measures ANOVA and Tukey's multiple comparisons test. Fentanyl caused a significant ($P < 0.0001$) time main effect ($F_{7,77} = 44.14$), dose main effect ($F_{3,33} = 98.57$), and time by dose interaction ($F_{21,231} = 11.16$) on percent

time spent in wakefulness (**Figure 3.4A**). Fentanyl produced a significant ($P < 0.0001$) time main effect ($F_{7, 77} = 40.1$), dose main effect ($F_{3, 33} = 97.8$), and time by dose interaction ($F_{21, 231} = 10.3$) on percent of time spent in NREM sleep (**Figure 3.4B**). The percent of the total recording time spent in REM sleep (**Figure 3.4C**) was significantly ($P < 0.0001$) changed by fentanyl, with a time main effect ($F_{7,77} = 20.85$), dose main effect ($F_{3,33} = 66.63$), and time by dose interaction ($F_{21,231} = 5.621$). The percent wakefulness (**Figure 3.4D**) was altered by morphine with a significant ($P < 0.0001$) time main effect ($F_{7,77} = 48.21$), dose main effect ($F_{3,33} = 139.2$), and time by dose interaction ($F_{21,231} = 13.16$). For NREM sleep (**Figure 3.4E**) morphine caused a significant ($P < 0.0001$) time main effect ($F_{7,77} = 46.86$), dose main effect ($F_{3,33} = 147.1$), and time by dose interaction ($F_{21,231} = 12.63$). For REM sleep (**Figure 3.4F**) morphine caused a significant ($P < 0.0001$) time main effect ($F_{7,77} = 28.24$), dose main effect ($F_{3,33} = 61.39$), and time by dose interaction ($F_{21,231} = 6.406$).

Opioid-induced increases in wakefulness were partially mediated by increased motor activity but not by changes in subcutaneous body temperature

Figure 3.5A plots the increase in motor activity caused by increasing doses of fentanyl and morphine relative to control (0 mg/kg). The ED_{50} values for the effects of fentanyl and morphine on motor activity were 0.10 and 5.88 mg/kg, respectively. One-way repeated measures ANOVA showed a significant ($P < 0.0001$) main effect of fentanyl ($F_{8,88} = 34.7$) and morphine ($F_{8,88} = 59.24$) on motor activity during wakefulness. Dunnett's multiple comparisons test revealed that doses of fentanyl ≥ 0.1 mg/kg and doses of morphine ≥ 3 mg/kg significantly (*$P < 0.001$) increased motor activity.

Within-subject multi-level mediation analysis was used to assess whether motor activity affected

the relationship between opioid dose and percent of time spent awake (**Figure 3.5B and 3.5C**). Opioid dose (mg/kg) was the independent variable hypothesized to be causal, percent of time spent in wakefulness (% Wake) was the main dependent measure, and motor activity was hypothesized to have mediated the effect of opioid dose on percent of the 4-h recording time spent in wakefulness. The upward pointing blue arrow labeled **(a)** at left of **Figure 3.5B** indicates that fentanyl doses of 0.03 mg/kg ($P = 0.042$) and ≥ 0.1 mg/kg ($P < 0.001$ for each dose) significantly increased motor activity. The downward pointing blue arrow labeled **(b)** at right of **Figure 3.5B** indicates a significant effect of motor activity on % Wake ($P < 0.001$). The horizontal blue arrow labeled **(c')** of **Figure 3.5B** illustrates the findings that when taking motor activity into account, fentanyl dose was a significant predictor of % Wake for doses of 0.001 mg/kg ($P = 0.005$), 0.003 mg/kg ($P = 0.012$), and ≥ 0.1 mg/kg ($P < 0.001$). For every 1% increase in motor activity, the model predicted a 0.5% increase in % Wake. A Sobel test determined significant mediation ($P < 0.001$) with 15.9% of the variance in % Wake mediated by motor activity.

The results of mediation analysis for effects of morphine (**Figure 3.5C**) revealed that doses of 3, 10, and 30 mg/kg significantly ($P < 0.0001$ for each dose) increased motor activity (**Figure 3.5C**, upward pointing red arrow labeled **(a)** at left). The downward pointing red arrow labeled **(b)** at right of **Figure 3.5C** identifies the significant effect of motor activity on %Wake ($P < 0.001$). The horizontal red arrow labeled **(c')** of **Figure 3.5C** shows that when motor activity was considered, morphine doses ≥ 1 mg/kg were significant predictors of % Wake ($P < 0.0001$ for each dose). For every 1% increase in motor activity, the model predicted a 0.8% increase in % Wake. A Sobel test determined significant mediation ($P < 0.0001$) with 21.7% of

the variance in % Wake mediated by motor activity.

Figure 3.5D plots the dose-dependent changes in Tsc during wakefulness caused by fentanyl and morphine. The ID50 value for the effect of fentanyl on Tsc was 2.78 mg/kg. The Tsc data for morphine did not fit the nonlinear regression model. Thus, the spline function in Prism was used to fit both curves without a model. One-way repeated measures ANOVA showed a significant ($P < 0.0001$) main effect of fentanyl ($F_{8,88} = 59.3$) and morphine ($F_{8,88} = 25.7$) on Tsc during wakefulness (**Figure 3.5D**). Dunnett's multiple comparisons test revealed that the two highest doses of fentanyl (1 and 3 mg/kg) significantly (*$P < 0.001$) decreased Tsc. Higher doses of morphine had a biphasic effect on body temperature. Dunnett's multiple comparisons test showed that morphine at 3 mg/kg significantly increased Tsc (*$P = 0.02$), and at 30 mg/kg morphine significantly decreased Tsc (*$P < 0.0001$).

Within-subject multi-level mediation regression analysis also was used to determine whether Tsc influenced the relationship between opioid dose and percent of time spent awake. As described above, opioid dose (mg/kg) was the independent variable hypothesized to be causal, percent of time spent in wakefulness (% Wake) was the main dependent measure, and Tsc was proposed to have mediated the effect of opioid dose on % Wake. In contrast to motor activity, changes in body temperature did not mediate the effect of fentanyl dose or morphine dose on wakefulness.

Figure 3.5E shows that fentanyl at doses of 1 and 3 mg/kg significantly ($P < 0.0001$) altered Tsc (upward pointing blue arrow labeled (**a**) at left), but there was no significant effect of Tsc on % Wake (downward pointing blue arrow labeled (**b**) at right). The horizontal blue arrow labeled (**c'**) of **Figure 3.5E** shows that when Tsc was included as a covariate in the analysis, fentanyl at doses ≥ 0.1 mg/kg significantly ($P < 0.001$) increased % Wake.

The upward pointing red arrow labeled (**a**) at left of **Figure 3.5F** shows the morphine doses that significantly altered Tsc, and the downward pointing red arrow labeled (**b**) at right reports a nonsignificant effect of Tsc on % Wake. The horizontal red arrow labeled (**c'**) of **Figure 3.5F** shows that when Tsc was considered in the analyses, morphine at doses ≥ 1 mg/kg significantly ($P < 0.001$) increased % Wake. In summary, mediation analyses revealed that the fentanyl- and morphine-induced increases in wakefulness were partially mediated (15.9% and 21.7%, respectively) by motor activity, but not by opioid-induced changes in Tsc.

Discussion

Four main findings shaped discussion of the results relative to the current literature. First, antinociceptive doses of acutely administered fentanyl and morphine disrupted sleep architecture by increasing wakefulness and reducing NREM sleep and REM sleep. Second, fentanyl and morphine differentially altered EEG power during wakefulness. Third, both fentanyl and morphine caused dissociations among sleep-wake states, Tsc, and motor activity by lowering Tsc while increasing wakefulness and motor activity. Fourth, sleep disruption was partially mediated by the opioid-induced increase in motor activity but not by opioid-induced changes in Tsc. Considered together, the foregoing results provide the first simultaneous quantification in B6 mice of dose-dependent disruption of sleep, changes in EEG power during wakefulness, increased motor activity, and hypothermia caused by fentanyl and morphine. Dose response studies are necessary for understanding how opioids impact sleep and closely related physiological variables. Clarifying how opioids alter interactions among sleep, EEG power, motor activity, and body temperature is an essential step for developing countermeasures to unwanted opioid effects.

Antinociceptive doses of fentanyl and morphine disrupt sleep architecture

Sleep architecture refers to the normal, predictable sequence of sleep states across the sleep period (Angarita et al.). Sleep fragmentation is a disruption of sleep architecture defined by the intrusion of short episodes of wakefulness during the sleep period (Mark K. Greenwald et al., 2021; Li et al.; Ramesh et al.). Sleep fragmentation disrupts sleep continuity and can result in severe health consequences (Thomas, 2024). Sleep architecture in mice (**Figure 3.2A**) and humans can be assessed by quantifying the amount of time spent in each state of sleep, latency to sleep onset, number and duration of NREM and REM episodes, and the number and type of transitions between states.

Opioids prescribed for the clinical management of pain commonly produce undesired effects that include disruption of sleep architecture (Akinnusi et al., 2024; Eacret et al., 2020; Mark K Greenwald et al., 2021; Ralph Lydic et al., 2022). Even a single dose of morphine significantly disrupts sleep architecture in healthy human adults (Dimsdale et al., 2007; Shaw et al., 2005). The sleep-disrupting effects of opioids exacerbate pain (Dalanon et al., 2021; Muncey et al., 2010; Roehrs et al., 2006) and sleep disruption contributes to poorer cardiometabolic outcomes (Altman et al., 2012) and longer recovery periods after brain injury (Fleming et al., 2020). Sleep disruption may also contribute to relapse of opioid use disorder (Fathi et al., 2020; Huhn & Finan, 2022; James et al., 2020; Ralph Lydic et al., 2022; Tripathi et al., 2020). The foregoing unwanted effects of opioids are produced by complex interactions among multiple neuronal networks involved in regulating sleep and pain (Haack et al., 2020), and on hypothesized relationships among sleep disruption, pain, and opioid craving during withdrawal (Mark K. Greenwald et al.). The present study was not designed to elucidate the mechanisms underlying

neuronal network interactions. Rather, one goal of this study was to address a gap in knowledge by quantifying the dose-dependent effects of acutely administered fentanyl and morphine on sleep.

This study also sought to determine whether there are doses of fentanyl and morphine that produce the desired effect of antinociception without causing the undesired effect of sleep disruption. A systematic review of the literature revealed no previous dose-response data comparing the effects of fentanyl and morphine on nociception and sleep-wake states in B6 mice. Dose-response data are an essential tool for identifying the lowest effective dose of a drug, the maximum effect dose, and for comparing the relative potency of two or more drugs. The present dose-response data revealed the novel finding that even the lowest effective, antinociceptive doses of fentanyl (0.1 mg/kg) and morphine (1 mg/kg) caused a significant increase in wakefulness and decrease in NREM sleep and REM sleep (Figure 3). Antinociceptive doses of both opioids also significantly decreased the number of state transitions (**Table 3.1.**). Thus, in B6 mice, diminishing nociception by administering opioids cannot be achieved without disrupting sleep architecture.

Many commonly used drugs, such as benzodiazepines and antidepressants, have different effects on NREM sleep than on REM sleep (Dhuna & Malkani, 2020; Hambrecht-Wiedbusch et al., 2010; Roehrs & Roth, 2010). In contrast, the dose-response curves reported here show that the same doses of fentanyl and morphine that increased wakefulness decreased both NREM and REM sleep. Across all dependent measures of sleep (**Figure 3.3**) fentanyl was approximately 14 times more potent than morphine for decreasing sleep. The findings that both opioids increased the duration of wakefulness episodes, decreased the number of episodes of all states, and

decreased transitions between behavioral states show that in B6 mice opioids significantly disrupt sleep architecture, but do not cause sleep fragmentation. Thus, these results also provide the first normative dose-response data that will help to interpret future comparative studies of B6 females, obese mice, and a range of transgenic mice.

Opioids differentially alter EEG power during wakefulness

EEG power is being investigated as a potential biomarker for desired and undesired effects of fentanyl (Balanza et al., 2022), as well as for early detection of cognitive decline in neurodegenerative diseases such as mild cognitive impairment, Alzheimer's disease (D'Atri et al., 2021; Reda et al., 2017), and Parkinson's disease (Wood et al., 2021). The potential predictive value of EEG power has been suggested as a possible biomarker for identifying and understanding interactions among depression, opioid use disorder, and sleep disruption (Reid et al., 2024).

The data reported here revealed differential, dose-dependent effects of fentanyl and morphine on EEG power in specific frequency bands (**Figure 3.2C**). Morphine and fentanyl are full agonists at mu opioid receptors (Comer & Cahill, 2019). However, fentanyl, but not morphine, also blocks alpha-1 adrenergic receptors, dopamine D4.4 and D1 receptors, and reuptake of neurotransmitters via the vesicular monoamine transporter 2 (Torralva et al., 2020). These differential receptor-mediated effects have been suggested to be the basis of the different clinical effects between fentanyl and morphine in humans (Torralva et al., 2020). The differential pharmacology of fentanyl relative to morphine and other mu opioid receptor agonists (Han et al., 2022; Kelly et al., 2021; Kuo et al., 2015) may account for the differential effects of fentanyl and morphine on EEG power in B6 mice.

Opioids obtund wakefulness in humans, in part, by increasing EEG delta power during wakefulness (Eacret et al., 2020; Greenwald & Roehrs, 2005; R. Lydic et al., 2022). Fentanyl, but not morphine, significantly increased delta power during wakefulness in B6 mice (Figure 2). This finding is important because it is consistent with the interpretation that during wakefulness after fentanyl administration, cognitive function is likely to be impaired. An increase in EEG delta power during wakefulness has been referred to as a paradoxical pharmacological dissociation (Frohlich et al., 2023), because high EEG delta power is a classic trait that defines slow wave sleep in humans (Greene & Frank, 2010) and mice (Franken et al., 1999; Valatx et al., 1972). EEG delta power increases with increased time awake and is a well-established marker of homeostatic sleep drive (Greene & Frank, 2010; Timofeev et al., 2020; Vyazovskiy et al., 2011). Fentanyl-induced increases in delta power have been demonstrated in human volunteers (Greenwald & Roehrs, 2005) and in surgical patients during anesthesia (Scott et al., 1991; Smith et al., 1984). In an operating room setting prior to the delivery of a general anesthetic, fentanyl increased delta power and that increase was associated with loss of consciousness (Balanza et al., 2022). Increased EEG delta power during wakefulness is present in patients with Alzheimer disease (D'Atri et al., 2021) and during an awake resting state with eyes closed in patients that have Parkinson's disease with dementia (Pal et al., 2020). Increased slow EEG frequencies during waking also occur in patients with obstructive sleep apnea, which is characterized by excessive daytime sleepiness, decreased vigilance, impaired cognition, and an increased risk of accidents (D'Rozario et al., 2017; Puskás et al., 2017).

Theta power and beta power were significantly increased by the highest dose of fentanyl whereas the highest dose of morphine decreased theta and beta power. This finding further

illustrates differential pharmacological effects of these two mu opioid receptor agonists. Increased theta band activity is also induced in humans with increasing doses of fentanyl (Balanza et al., 2022; Greenwald & Roehrs, 2005). In humans increased theta power during wakefulness is associated with sleepiness and diminished performance on cognitive and psychomotor vigilance tasks (Zhao et al., 2021). Patients with Alzheimer's disease show an increase in awake, resting state theta power (Babiloni et al., 2021). In mice EEG beta power during active wakefulness occurs during processing of sensory information, whereas beta activity during quiet wakefulness signifies homeostatic sleep drive (Grønli et al., 2016). Increased EEG beta activity is a sign of central nervous system arousal in patients with insomnia (Perlis et al., 2001). Increased theta and beta power during wakefulness has been interpreted to indicate sleepiness and cortical hyperarousal occurring simultaneously in patients with insomnia (Zhao et al., 2021). Increased theta power during waking also occurs in patients with obstructive sleep apnea (D'Rozario et al., 2017).

Alpha power was significantly decreased in mice that received antinociceptive doses of fentanyl and morphine. In humans the EEG alpha rhythm characterizes quiet wakefulness with eyes closed, decreases in amplitude when eyes are opened, and contributes to cognitive processing (reviewed in Bazanova & Vernon, 2014). Alpha band activity in healthy humans has been shown to reflect attention focused on spatial locations that are maintained in working memory (Sutterer et al., 2021). Resting state alpha power is decreased in patients with mild cognitive impairment and is progressively reduced with increasing impairment (Lejkoa et al., 2020). EEG alpha power in the evening is decreased relative to healthy controls in patients with Alzheimer's disease and mild cognitive impairment (D'Atri et al., 2021).

In summary, these novel data from B6 mice show that higher doses of fentanyl significantly increased EEG power in delta, theta, and beta bands and significantly decreased EEG alpha power. Morphine had no effect on EEG delta power and at higher doses significantly decreased EEG power in theta, alpha, and beta bands. Many of these results in mice are similar to opioid-induced EEG change in humans, supporting the use of B6 mice as a model for adverse opioid effects. These findings in mice support the interpretation that fentanyl produces a dissociated state of wakefulness that is not compatible with optimal cortical functioning. The dose-response data reported here encourage the future use of high density EEG in mice (Wasilczuk et al., 2016) in order to more completely understand these adverse opioid effects.

Endogenous changes in mouse body temperature

Body temperature exhibits a circadian rhythm and sleep onset in humans occurs during the maximal rate of decline in body temperature (Campbell & Broughton, 1994; Czeisler et al., 1980). Body temperature also fluctuates during sleep (Glotzbach & Heller, 1976) and is lower during NREM sleep than during wakefulness in birds and mammals (Heller, 1988). **Figure 3.1A** shows the expected decrease in Tsc during NREM sleep compared with wakefulness under control (saline injection) conditions. Tsc also decreased below waking and NREM sleep levels during REM sleep, which is consistent with the finding that all mammals studied to date do not regulate their body temperature during REM sleep (Glotzbach & Heller, 1976; Heller, 1988; Jhaveri et al., 2007). Regarding effects of ambient temperature on mouse body temperature, the B6 mice in the present study were housed at temperatures that did not significantly change NREM sleep or REM sleep during the light period (Jhaveri et al., 2007).

Opioids decreased Tsc while increasing motor activity and wakefulness

Tsc did not show a classic sigmoidal, agonist dose response to morphine (**Figure 3.5D**). Both opioids at higher doses significantly decreased Tsc despite causing concomitant, significant increases in locomotor activity (**Figure 3.5A**). Motor activity is thermogenic (Abreu-Vieira et al., 2015) and normally precedes an increase in mouse body temperature (Lateef et al., 2014). Morphine had a biphasic effect, significantly increasing Tsc at a dose of 3 mg/kg and decreasing Tsc at the 30 mg/kg dose. Fentanyl produced only hypothermia, decreasing Tsc at doses of 1 and 3 mg/kg. These dose-dependent effects on Tsc are similar to rectal temperature changes reported for NMRI mice in response to similar doses of morphine and fentanyl (Baker & Meert, 2002). The results show that fentanyl dissociated the normal physiological relationship of motor activity to body temperature.

Dose-dependent increases in motor activity in B6 mice caused by fentanyl and morphine have been documented (Olson et al., 2023; Santos et al., 2022; Varshneya et al., 2023). Assays of motor activity typically involve administering a drug before placing a mouse in a novel environment and quantifying the number of times an infrared beam is broken and/or distance traveled (Fowler, 2010). The present study measured states of sleep and wakefulness, motor activity, and Tsc simultaneously via telemetry while mice remained in their home cages. This approach avoided causing the increase in exploratory behavior that results in sleep disruption when mice are placed in a novel environment (Tang et al., 2005).

A recent study of opioid-induced respiratory depression in mice reviewed differences between spontaneous and opioid-induced locomotor activity, noting that after opioid administration periods of "automatic locomotion" alternate with periods when mice are immobile (Haouzi et al., 2021). This alternating pattern of locomotor activity and immobility was observed in the present

experiments.

The lowest effective doses of fentanyl (0.1 mg/kg) and morphine (1 mg/kg) that increased motor activity (**Figure 3.5A**) were also the lowest effective doses for disrupting sleep (**Figure 3.3A-C**). These data suggest that opioids disrupt interactions among neural circuits regulating body temperature, motor activity, and states of sleep and wakefulness. These findings also raised the question of whether sleep disruption following opioid administration resulted from increased motor activity rather than an effect on sleep-wake generating mechanisms. That question was addressed using mediation analysis.

Opioid-induced sleep disruption was partially mediated by motor activity but not by body temperature

Mediation analysis is a statistical method used to quantify the extent to which intervening variables (mediators) affect an outcome (Vuorre & Bolger, 2018). Mediation analysis was used to determine whether opioid-induced changes in motor activity and/or Tsc contributed to the effects of fentanyl or morphine dose on the opioid-induced increase in wakefulness. These analyses showed that the increases in motor activity induced by specific doses of fentanyl and morphine mediated a portion of the opioid-induced increase in wakefulness (**Figure 3.5**). Mediation analysis also revealed that although both opioids significantly changed body temperature, those changes did not mediate a significant portion of the opioid-induced increases in wakefulness.

Limitations, future directions, and conclusions

The data reported here were limited to male B6 mice and focused on fentanyl as a widely used drug of abuse, and morphine as the gold standard for mu opioid receptor mediated effects. These

results encourage future studies that use female B6 mice to characterize sex-specific and dose-dependent effects of fentanyl, morphine, and opioids used to treat opioid use disorder, such as methadone and buprenorphine (Angel et al., 2018; Glovak et al., 2022). Future studies of chronic opioid administration are needed to determine whether tolerance develops to the sleep-disruptive effects of chronic opioid administration in mice. In patients taking methadone or buprenorphine for medication assisted therapy REM sleep is suppressed and daytime sleepiness has been reported (Mark K. Greenwald et al.). Dose-related effects of chronic opioid administration on multiple, simultaneously collected physiological variables will provide insights into how chronically used opioids affect sleep and interacting physiological control systems. These results from eight doses of fentanyl and morphine spanning 4 log units provide data not previously available to guide the design of future studies.

In conclusion, the present data quantify similarities and differences between the effects of opioids on sleep states and neurobehavioral traits in mice and humans. Similarities include sleep disruption, altered EEG power, hyperthermia, and hypothermia. An important species-specific difference is increased motor activity in mice. This study also demonstrated that mediation analyses can differentiate the simultaneous effects of opioids on multiple dependent variables. The contribution of validated animal models to research on substance use disorders is well recognized (Kamens et al., 2023). The novelty of the current findings is relevant to preclinical studies using mice to inform data-based treatment of pain, addiction, and sleep disorders.

References

Abreu-Vieira, G., Xiao, C., Gavrilova, O., & Reitman, M. L. (2015). Integration of body temperature into the analysis of energy expenditure in the mouse. *Molecular Metabolism, 4*(6): 461-470. https://doi.org/10.1016/j.molmet.2015.03.001

Akinnusi, M., Martinson, A., & El-Solh, A. A. (2024). Treatment of insomnia associated with alcohol and opioid use: A narrative review. *Sleep and Biological Rhythms*. https://doi.org/10.1007/s41105-024-00544-x

Altman, N. G., Izci-Balserak, B., Schopfer, E., Jackson, N., Rattanaumpawan, P., Gehrman, P. R., Patel, N. P., & Grandner, M. A. (2012). Sleep duration versus sleep insufficiency as predictors of cardiometabolic health outcomes. *Sleep Medicine, 13*(10): 1261-1270. https://doi.org/10.1016/j.sleep.2012.08.005

Anderson, E. J., & Jeter, J. P. (2023). Dermatological implications of sleep deprivation in the us military. *Cutis, 111*(3): 146-149. https://doi.org/10.12788/cutis.0723

Angarita, G. A., Emadi, N., Hodges, S., & Morgan, P. T. (2016). Sleep abnormalities associated with alcohol, cannabis, cocaine, and opiate use: A comprehensive review. *Addiction Science & Clinical Practice, 11*(1): 9. https://doi.org/10.1186/s13722-016-0056-7

Angel, T. C., Glovak, A. Z., Alami, A. W., Mihalko, A. S., Price, A. J., Jiang, A. Y., Baghdoyan, H. A., & Lydic, A. R. (2018). Buprenorphine depresses respiratory variability in obese mice with altered leptin signaling. *Anesthesiology, 128*(5): 984-991. https://doi.org/10.1097/ALN.0000000000002073

Babiloni, C., Arakaki, X., Azami, H., Bennys, K., Blinowska, K., Bonanni, L., Bujan, A., Carrillo, M. C., Cichocki, A., Frutos-Lucas, J. d., Percio, C. D., Dubois, B., Edelmayer, R., Egan,

G., Epelbaum, S., Escudero, J., Evans, A., Farina, F., Fargo, K., . . . Guntekin, B. (2021). Measures of resting state eeg rhythms for clinical trials in alzheimer's disease: Recommendations of an expert panel. *Alzheimer's and Dementia*, *17*(9): 1528-1553. https://doi.org/10.1002/alz.12311

Baker, A. K., & Meert, T. F. (2002). Functional effects of systemically administered agonists and antagonists of μ, δ, and κ opioid receptor subtypes on body temperature in mice. *The Journal of Pharmacology and Experimental Therapeutics*, *302*(3): 1253-1264. https://doi.org/10.1124/jpet.102.037655

Balanza, G. A., Bharadwaj, K. M., Mullen, A. C., Beck, A., C.Work, E., McGovern, F. J., Houle, T. T., Eric, T. P., & Purdon, P. L. (2022). An electroencephalogram biomarker of fentanyl drug effects. *Proceedings of the National Academy of Sciences Nexus*, *1*(4): 1-7. https://doi.org/10.1093/pnasnexus/pgac158

Bazanova, O. M., & Vernon, D. (2014). Interpreting eeg alpha activity. *Neuroscience and Biobehavioral Reviews*, *44*(7): 94-110. https://doi.org/10.1016/j.neubiorev.2013.05.007

Brower, K. J., & Perron, B. E. (2010). Sleep disturbance as a universal risk factor for relapse in addictions to psychoactive substances. *Medical Hypotheses*, *74*(5): 928-933. https://doi.org/10.1016/j.mehy.2009.10.020

Campbell, S. S., & Broughton, R. J. (1994). Rapid decline in body temperature before sleep: Fluffing the physiological pillow? *Chronobiology International*, *11*(2): 126-131. https://doi.org/10.3109/07420529409055899

Cao, M., & Javaheri, S. (2018). Effects of chronic opioid use on sleep and wake. *Sleep Medicine Clinics*, *13*(2): 271-281. https://doi.org/10.1016/j.jsmc.2018.02.002

Comer, S. D., & Cahill, C. M. (2019). Fentanyl: Receptor pharmacology, abuse potential, and implications for treatment. *Neuroscience & Biobehavioral Reviews, 106*(11): 49-57. https://doi.org/10.1016/j.neubiorev.2018.12.005

Czeisler, C. A., Weitzman, E. D., Moore-Ede, M. C., Zimmerman, J. C., & Knauer, R. S. (1980). Human sleep: Its duration and organization depend on its circadian phase. *Science, 210*(4475): 1264-1267. https://doi.org/10.1126/science.7434029

D'Rozario, A. L., Cross, N. E., Vakulin, A., Bartlett, D. J., Wong, K. K. H., Wang, D., & Grunstein, R. R. (2017). Quantitative electroencephalogram measures in adult obstructive sleep apnea – potential biomarkers of neurobehavioural functioning. *Sleep Medicine Reviews, 36*(12): 29-42. https://doi.org/10.1016/j.smrv.2016.10.003

D'Atri, A., Scarpelli, S., Gorgoni, M., Truglia, I., Lauri, G., Cordone, S., Ferrara, M., Marra, C., Rossini, P. M., & Gennaro, L. D. (2021). Eeg alterations during wake and sleep in mild cognitive impairment and alzheimer's disease. *iScience, 24*(102386): 1-16. https://doi.org/10.1016/j.isci.2021.102386

Dahlhamer, J., Lucas, J., Zelaya, C., Nahin, R., Mackey, S., DeBar, L., Kerns, R., Von Korff, M., Porter, L., & Helmick, C. (2018). Prevalence of chronic pain and high-impact chronic pain among adults - united states, 2016. *MMWR Morbidity and Mortality Weekly Report, 67*(36): 1001-1006. https://doi.org/10.15585/mmwr.mm6736a2

Dalanon, J., Chikahisa, S., Shiuchi, T., Shimizu, N., Chavan, P., Suzuki, Y., Okura, K., Séi, H., & Matsuka, Y. (2021). Pain sensitivity increases with sleep disturbance under predictable chronic mild stress in mice. *Scientific Reports, 11*(1): 14231. https://doi.org/10.1038/s41598-021-93560-7

Dhuna, N. A., & Malkani, R. G. (2020). Antidepressants and their impact on sleep. *Current Sleep Medicine Reports, 6*: 216-225. https://doi.org/10.1007/s40675-020-00189-5

Dimsdale, J. E., Norman, D., DeJardin, D., & Wallace, M. S. (2007). The effect of opioids on sleep architecture. *Journal of clinical sleep medicine, 3*(1): 33-36. https://doi.org/10.5664/jcsm.26742

Dowell, D., Ragan, K., Jones, C., Baldwin, G., & Chou, R. (2022). Cdc clinical practice guideline for prescribing opioids for pain — united states. *Morbidity and Mortality Weekly Report, 71*(No. RR-3): 1-95. https://doi.org/10.15585/mmwr.rr7103a1

Eacret, D., Veasey, S. C., & Blendy, J. A. (2020). Bidirectional relationship between opioids and disrupted sleep: Putative mechanisms. *Molecular Pharmacology, 98*(4): 445-453. https://doi.org/ 10.1124/mol.119.119107

Fathi, H. R., Yoonessi, A., Khatibi, A., Rezaeitalab, F., & Rezaei-Ardani, A. (2020). Crosstalk between sleep disturbance and opioid use disorder: A narrative review. *Addiction & Health, 12*(2): 140-158. https://doi.org/10.22122/ahj.v12i2.249

Finan, P. H., Goodin, B. R., & Smith, M. T. (2013). The association of sleep and pain: An apdate and a path forward. *The Journal of Pain, 14*(12): 1539-1552. https://doi.org/10.1016/j.jpain.2013.08.007

Fleming, M. K., Smejka, T., Henderson Slater, D., Van Gils, V., Garratt, E., Yilmaz Kara, E., & Johansen-Berg, H. (2020). Sleep disruption after brain injury is associated with worse motor outcomes and slower functional recovery. *Neurorehabilitation and Neural Repair, 34*(7): 661-671. https://doi.org/10.1177/1545968320929669

Fletcher, D., & Martinez, V. (2014). Opioid-induced hyperalgesia in patients after surgery: A

systematic review and a meta-analysis. *British Journal of Anaesthesia, 112*(6): 991-1004. https://doi.org/10.1093/bja/aeu137

Fowler, S. C. (2010). Motor activity and stereotypy. In I. P. Stolerman (Ed.), *Encyclopedia of psychopharmacology* (pp. 799-805). Springer. https://doi.org/10.1007/978-3-540-68706-1_279

Franken, P., Malafosse, A., & Tafti, M. (1999). Genetics determinants of sleep in regulation in inbred mice. *Sleep, 22*(2): 155-169. https://doi.org/10.1093/sleep/22.2.155

Frohlich, J., Mediano, P. A. M., Bavato, F., & Gharabaghi, A. (2023). Paradoxical pharmacological dissociations result from drugs that enhance delta oscillations but preserve consciousness. *Communications Biology, 6*(654): 1-10. https://doi.org/10.1038/s42003-023-04988-8

Glotzbach, S. F., & Heller, H. C. (1976). Central nervous system regulation of body temperature during sleep. *Science, 194*(4264): 537-539. https://doi.org/10.1126/science.973138

Glovak, A. Z., Angel, C., O'Brien, C. B., Baghdoyan, H. A., & Lydic, R. (2022). Buprenorphine differentially alters breathing among four congenic mouse lines as a function of dose, sex, and leptin status. *Respiratory Physiology & Neurobiology, 297*(103834): 1-9. https://doi.org/10.1016/j.resp.2021.103834

Greene, R. W., & Frank, M. G. (2010). Slow wave activity during sleep: Functional and therapeutic implications. *The Neuroscientist, 16*(6): 618-633. https://doi.org/10.1177/1073858410377064

Greenwald, M. K., Moses, T. E. H., & Roehrs, T. A. (2021). At the intersection of sleep deficiency and opioid use: Mechanisms and therapeutic opportunities. *Translational Research, 234*:

58-73. https://doi.org/10.1016/j.trsl.2021.03.006

Greenwald, M. K., & Roehrs, T. A. (2005). Mu-opioid self-administration vs passive administration in heroin abusers produces differential eeg activation. *Neuropsychopharmacology*, *30*(1): 212-221. https://doi.org/10.1038/sj.npp.1300596

Grønli, J., Rempe, M. J., Clerern, W. C., Schmidt, M., & Wisor, J. P. (2016). Beta eeg reflects sensory processing in active wakefulness and homeostatic sleep drive in quiet wakefulness. *Journal of Sleep Research*, *25*(3): 257-268. https://doi.org/10.1111/jsr.12380

Haack, M., Simpson, N., Sethna, N., Kaur, S., & Mullington, J. (2020). Sleep deficiency and chronic pain: Potential underlying mechanisms and clinical implications. *Neuropsychopharmacology*, *45*(1): 205-216. https://doi.org/10.1038/s41386-019-0439-z

Hambrecht-Wiedbusch, V. S., Gauthier, E. A., Baghdoyan, H. A., & Lydic, R. (2010). Benzodiazepine receptor agonists cause drug-specific and state-specific alterations in eeg power and acetylcholine release in rat pontine reticular formation. *Sleep*, *33*(7): 909-918. https://doi.org/10.1093/sleep/33.7.909

Han, Y., Cao, L., Yuan, K., Shi, J., Yan, W., & Lu, L. (2022). Unique pharmacology, brain dysfunction, and therapeurtic advancements for fentanyl misuse and abuse. *Neuroscience Bulletin*, *38*(11): 1365-1382. https://doi.org/10.1007/s12264-022-00872-3

Haouzi, P., McCann, M., & Tubbs, N. (2021). Respiratory effects of low and high doses of fentanyl in control and β-arrestin 2-deficient mice. *Journal of Neurophysiology*, *125*(4): 1396-1407. https://doi.org/10.1152/jn.00711.2020

Heller, H. C. (1988). Sleep and hypometabolism. *Canadian Journal of Zoology*, *66*(1): 61-69. https://doi.org/10.1139/z88-008

Huhn, A. S., & Finan, P. H. (2022). Sleep disturbance as a therapeutic target to improve opioid use disorder treatment. *Experimental and Clinical Psychopharmacology*, *30*(6): 1024-1035. https://doi.org/10.1037/pha0000477

James, M. H., Fragale, J. E., Aurora, R. N., Cooperman, N. A., Langleben, D. D., & Aston-Jones, G. (2020). Repurposing the dual orexin receptor antagonist suvorexant for the treatment of opioid use disorder: Why sleep on this any longer? *Neuropsychopharmacology*, *45*(5): 717-791. https://doi.org/10.1038/s41386-020-0619-x

Jhaveri, K. A., Trammell, R. A., & Toth, L. A. (2007). Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of c57bl/6j mice. *Brain, Behavior, and Immunity*, *21*(7): 975-987. https://doi.org/10.1016/j.bbi.2007.03.007

Kamens, H. M., Cramer, S., Hanley, R. N., Chase, S., Wickenheisser, A., Horton, W. J., & Zhang, N. (2023). Neuroimaging of opioid exposure: A review of preclinical animal models to inform addiction research. *Psychopharmacology*, *240*(12): 2459-2482. https://doi.org/10.1007/s00213-023-06477-6

Kelly, E., Sutcliffe, K., Cavallo, D., Ramos-Gonzalez, N., Alhosan, N., & Henderson, G. (2021). The anomalous pharmacology of fentanyl. *British Journal of Pharmacology*, *180*(7): 797-812. https://doi.org/10.1111/bph.15573

Knutson, K. (2015). Sleep and pain: Summary of the 2015 sleep in america poll. *Sleep Health*, *1*(2): 85. https://doi.org/10.1016/j.sleh.2015.03.006

Kuo, A., Wyse, B. D., Meutermans, W., & Smith, M. T. (2015). In vivo profiling of seven common opioids for antinociception, constipation and respiratory depression: No two opioids have the same profile. *British Journal of Pharmacology*, *172*(2): 532-548.

https://doi.org/10.1111/bph.12696

Lateef, D. M., Abreu-Vieira, G., Xiao, C., & Reitman, M. L. (2014). Regulation of body temperature and brown adipose tissue thermogenesis by bombesin receptor subtype-3. *American Journal of Physiology. Endocrinology and Metabolism*, *306*(6): E681–E687. https://doi.org/10.1152/ajpendo.00615.2013

Lejkoa, N., Larabia, D. I., Herrmannd, C. S., Alemana, A., & Ćurčić-Blakea, B. (2020). Alpha power and functional connectivity in cognitive decline: A systematic review and meta-analysis. *Journal of Alzheimer's Disease*, *78*(3): 1047-1088. https://doi.org/10.3233/JAD-200962

Li, S.-B., Damonte, V. M., Chen, C., Wang, G. X., Kebschull, J. M., Yamaguchi, H., Bian, W.-J., Purmann, C., Pattni, R., Urban, A. E., Mourrain, P., Kauer, J. A., Scherrer, G., & Lecea, L. d. (2022). Hyperexcitable arousal circuits drive sleep instability during aging. *Science, 375*(eabh3021): 1-14. https://doi.org/10.1126/science.abh3021

López-Muciño, L. A., García-García, F., Cueto-Escobedo, J., Acosta-Hernández, M., Venebra-Muñoz, A., & Rodríguez-Alba, J. C. (2022). Sleep loss and addiction. *Neuroscience & Biobehavioral Reviews*, *141*(104832): 1-11 https://doi.org/10.1016/j.neubiorev.2022.104832

Lydic, R., Hillman, D., Jiang, Y., Baghdoyan, H. A., & O'Brien, C. B. (2022). Opiate actions on sleep and breathing. In M. H. Kryger, T. Roth, & C. A. Goldstein (Eds.), *Principles and practice of sleep medicine* (7th edition. ed., Vol. 1, pp. 510-518). Elsevier, New York.

Lydic, R., Hillman, D. R., Jiang, Y., Baghdoyan, H. A., & O'Brien, C. O. (2022). Opioid actions on sleep and breathing. In M. H. Kryger, T. Roth, & C. A. Goldstein (Eds.), *Principles and*

practice of sleep medicine (Seventh ed., Vol. 1, pp. 510-518). Elsevier.

McLain, J. M., Alami, W. H., Glovak, Z. T., Cooley, C. R., Burke, S. J., Collier, J. J., Baghdoyan, H. A., Karlstad, M. D., & Lydic, R. (2018). Sleep fragmentation delays wound healing in a mouse model of type 2 diabetes. *Sleep*, *41*(11): 1-9. https://doi.org/10.1093/sleep/zsy156

Muncey, A. R., Saulles, A. R., Koch, L. G., Britton, S. L., Baghdoyan, H. A., & Lydic, R. (2010). Disrupted sleep and delayed recovery from chronic peripheral neuropathy are distinct phenotypes in a rat model of metabolic syndrome. *Anesthesiology, 113*(5): 1176-1185. https://doi.org/10.1097/ALN.0b013e3181f56248

Olson, K. M., Hillhouse, T. M., Burgess, G. E., West, J. L., Hallahan, J. E., Dripps, I. J., Ladetto, A. G., Rice, K. C., Jutkiewicz, E. M., & Traynor, J. R. (2023). Delta opioid receptor-mediated antidepressant-like effects of diprenorphine in mice. *The Journal of Pharmacolopgy and Experimental Therapeutics, 384*(3): 343-352. https://doi.org/10.1124/jpet.122.001182

Pal, A., Pegwal, N., Behari, M., & Sharma, R. (2020). High delta and gamma eeg power in resting state characterise dementia in parkinson's patients. *Biomarkers in Neuropsychiatry*, *3*(100027): 1-5. https://doi.org/10.1016/j.bionps.2020.100027

Perlis, M. L., Merica, H., Smith, M. T., & Giles, D. E. (2001). Beta eeg activity and insomnia. *Sleep Medicine Reviews*, *5*(5): 365-376. https://doi.org/10.1053/smrv.2001.0151

Puskás, S., Kozák, N., Sulina, D., Csiba, L., & Magyar, M. T. n. (2017). Quantitative eeg in obstructive sleep apnea syndrome: A review of the literature. *Reviews in the Neurosciences, 28*(3): 265-270. https://doi.org/10.1515/revneuro-2016-0064

Ramesh, V., Kaushal, N., & Gozal, D. (2009). Sleep fragmentation modifies eeg delta power

during slow wave sleep in socially isolated and paired mice. *Sleep Science*, *2*(2): 64-75.

Ramirez, J. M., Burgraff, N. J., Wei, A. D., Baertsch, N. A., Varga, A. G., Baghdoyan, H. A., Lydic, R., Morris, K. F., Bolser, D. C., & Levitt, E. S. (2021). Neuronal mechanisms underlying opioid-induced respiratory depression: Our current understanding. *Journal of Neurophysiology*, *125*(5): 1899-1919. https://doi.org/10.1152/jn.00017.2021

Reda, F., Gorgoni, M., Lauri, G., Truglia, I., Cordone, S., Scarpelli, S., Mangiaruga, A., D'Atri, A., Ferrara, M., Lacidogna, G., Marra, C., Rossini, P. M., & Gennaro, L. D. (2017). In search of sleep biomarkers of alzheimer's disease: K-complexes do not discriminate between patients with mild cognitive impairment and healthy controls. *Brain Sciences*, *7*(51): 1-13. https://doi.org/10.3390/brainsci7050051

Reid, M. J., Dunn, K. E., Abraham, L., Ellis, J., Hunt, C., Gamaldo, C. E., Coon, W. G., Mun, C. J., Strain, E. C., Smith, M. T., Finan, P. H., & Huhn, A. S. (2024). Suvorexant alters dynamics of the sleep-electroencephalography-power spectrum and depressive-symptom trajectories during inpatient opioid withdrawal. *Sleep*, *47*(4): 1-15. https://doi.org/10.1093/sleep/zsae025

Roehrs, T., Hyde, M., Blaisdell, B., Greenwald, M., & Roth, T. (2006). Sleep loss and rem sleep loss are hyperalgesic. *Sleep*, *29*(2): 145-151. https://doi.org/10.1093/sleep/29.2.145

Roehrs, T., & Roth, T. (2010). Drug-related sleep stage changes: Functional significance and clinical relevance. *Sleep Medicine Clinics*, *5*(4): 559-570. https://doi.org/10.1016/j.jsmc.2010.08.002

Roehrs, T., Sibai, M., & Roth, T. (2021). Sleep and alertness disturbance and substance use disorders: A bi-directional relation. *Pharmacology, Biochemistry and Behavior*,

203(173153): 1-8. https://doi.org/10.1016/j.pbb.2021.173153

Santos, E. J., Banks, M. L., & Negus, S. S. (2022). Role of efficacy as a determinant of locomotor activation by mu opioid receptor ligands in female and male mice. *Journal of Pharmacology and Experimental Therapeutics*, *382*(1): 44-53. https://doi.org/10.1124/jpet.121.001045

Scott, J. C., Cooke, J. E., & Stanski, D. R. (1991). Electroencephalographic quantification of opioid effect: Comparative pharmacodynamics of fentanyl and sufentanil. *Anesthesiology*, *74*(1): 34-43. https://doi.org/10.1097/00000542-199101000-00007

Shaw, I. R., Lavigne, G., Mayer, P., & Choinière, M. (2005). Acute intravenous administration of morphine perturbs sleep architecture in healthy pain-free young adults: A preliminary study. *Sleep*, *28*(6): 677-682. https://doi.org/10.1093/sleep/28.6.677

Siegel, J. M. (2022). Sleep function: An evolutionary perspective. *Lancet Neurol*, *21*(10): 937-946. https://doi.org/10.1016/S1474-4422(22)00210-1

Smith, N. T., Dec-Silver, H., Sanford, T. J. J., Westover, C. J. J., Quinn, M. L., Klein, F., & DA, D. (1984). Eegs during high-dose fentanyl-, sufentanil-, or morphine-oxygen anesthesia. *Anesthesia & Analgesia*, *63*(4): 386-393.

Sun, Y., Tisdale, R. K., Yamashita, A., & Kilduff, T. S. (2023). Peripheral vs. Core body temperature as hypocretin/orexin neurons degenerate: Exercise mitigates increased heat loss. *Peptides*, *164*(171002): 1-10. https://doi.org/10.1016/j.peptides.2023.171002

Sutterer, D. W., Polyn, S. M., & Woodman, G. F. (2021). Alpha-band activity tracks a two-dimensional spotlight of attention during spatial working memory maintenance. *Journal of Neurophysiology*, *125*(3): 957-971. https://doi.org/10.1152/jn.00582.2020

Tang, X., Xiao, J., Parris, B. S., Fang, J., & Sanford, L. D. (2005). Differential effects of two types of environmental novelty on activity and sleep in balb/cj and c57bl/6j mice. *Physiology & Behavior*, *85*(4): 419-429. https://doi.org/10.1016/j.physbeh.2005.05.008

Thomas, R. J. (2024). A matter of fragmentation. *Sleep*, *47*(4): 1-4. https://doi.org/10.1093/sleep/zsae030

Timofeev, I., Schoch, S. F., LeBourgeois, M. K., Huber, R., Riedner, B. A., & Kurth, S. (2020). Spatio-temporal properties of sleep slow waves and implications for development. *Current Opinion in Physiology*, *15*(1): 172-182. https://doi.org/10.1016/j.cophys.2020.01.007

Torralva, R., Eshleman, A. J., Swanson, T. L., Schmachtenberg, J. L., Schutzer, W. E., Bloom, S. H., Wolfrum, K. M., Reed, J. F., & Janowsky, A. (2020). Fentanyl but not morphine interacts with nonopioid recombinant human neurotransmitter receptors and transporters. *Journal of Pharmacology and Experimental Therapeutics*, *374*(3): 376-391. https://doi.org/10.1124/jpet.120.265561

Tripathi, R., Rao, R., Dhawan, A., Jain, R., & Sinha, S. (2020). Opioids and sleep - a review of literature. *Sleep Medicine*, *67*(3): 269-275. https://doi.org/10.1016/j.sleep.2019.06.012

Valatx, J. L., Bugat, R., & Jouvet, M. (1972). Genetic studies of sleep in mice. *Nature*, *238*(5361): 226-227. https://doi.org/10.1038/238226a0

Vanini, G., Nemanis, K., Baghdoyan, H. A., & Lydic, R. (2014). Gabaergic transmission in rat pontine reticular formation regulates the induction phase of anesthesia and modulates hyperalgesia caused by sleep deprivation. *European Journal of Neuroscience*, *40*(1): 2264-2273. https://doi.org/10.1111/ejn.12571

Varshneya, N. B., Walentiny, D. M., Stevens, D. L., Walker, T. D., Akinfiresoye, L. R., &

Beardsley, P. M. (2023). Structurally diverse fentanyl analogs yield differential locomotor activities in mice. *Pharmacology Biochemistry and Behavior, 222*(173496): 1-7. https://doi.org/10.1016/j.pbb.2022.173496

Volkow, N. D., & McLellan, A. T. (2016). Opioid abuse in chronic pain — misconceptions and mitigation strategies. *New England Journal of Medicine, 374*(13): 1253-1263. https://doi.org/10.1056/NEJMra1507771

Vuorre, M., & Bolger, N. (2018). Within-subject mediation analysis for experimental data in cognitive psychology and neuroscience. *Behavior Research Methods, 50*(5): 2125-2143. https://doi.org/10.3758/s13428-017-0980-9

Vyazovskiy, V. V., Olcese, U., Hanlon, E. C., Nir, Y., Cirelli, C., & Tononi, G. (2011). Local sleep in awake rats. *Nature, 472*(7344): 443-447. https://doi.org/10.1038/nature10009

Wasilczuk, A. Z., Proekt, A., Kelz, M. B., & McKinstry-Wu, A. R. (2016). High-density electroencephalographic acquisition in a rodent model using low-cost and open-source resources. *Journal of Visualized Experiments, 117*(e54908): 1-10. https://doi.org/10.3791/54908

Wood, K. H., Memon, A. A., Memon, R. A., Joop, A., Pilkington, J., Catiul, C., Gerstenecker, A., Triebel, K., Cutter, G., Bamman, M. M., Miocinovic, S., & Amara, A. W. (2021). Slow wave sleep and eeg delta spectral power are associated with cognitive function in parkinson's disease. *Journal of Parkinsons Disease, 11*(2): 703-714. https://doi.org/10.3233/JPD-202215

Yamazaki, E. M., Antler, C. A., Lasek, C. R., & Goel, N. (2021). Residual, differential neurobehavioral deficits linger after multiple recovery nights following chronic sleep

restriction or acute total sleep deprivation. *Sleep*, *44*(4): zsaa224. https://doi.org/10.1093/sleep/zsaa224

Zhao, W., Someren, E. J. W. V., Li, C., Chen, X., Gui, W., Tian, Y., Liu, Y., & Lei, X. (2021). Eeg spectral analysis in insomnia disorder: A systematic review and meta-analysis. *Sleep Medicine Reviews*, *59*(101457): 1-15. https://doi.org/10.1016/j.smrv.2021.101457

Appendix

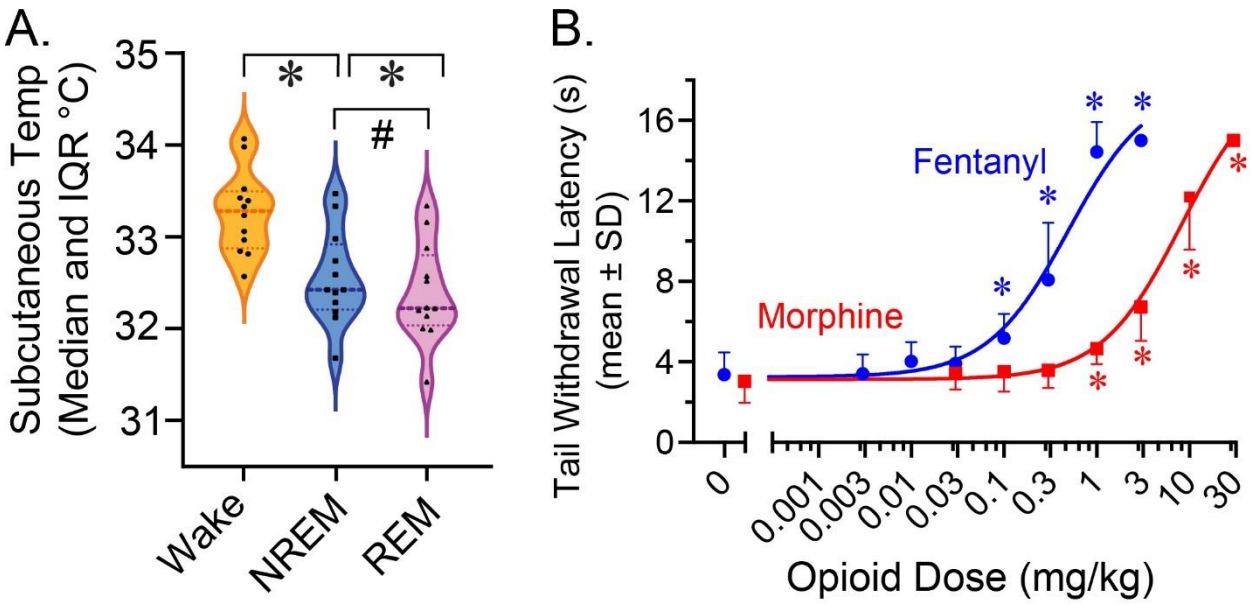


Figure 3.1. Subcutaneous body temperature (Tsc) and opioid-induced antinociception in C57BL/6J mice.
(A) Tsc was averaged across the 4-h recording periods during wakefulness (orange), NREM sleep (blue), and REM sleep (purple) after administration of saline (n=12 opioid naïve mice). Black filled symbols plot the mean Tsc of each mouse. The dashed, horizontal line within each violin shows median Tsc for all mice. The thin dotted lines mark the 25th and 75th percentile of each distribution. One-way repeated measures ANOVA and Tukey's multiple comparisons test revealed a significant (*P<0.0001) decrease in Tsc during NREM sleep and REM sleep relative to wakefulness, and during REM sleep relative to NREM sleep (#P=0.0052). (B) Tail withdrawal latency was measured in 12 additional mice after administration of saline (0 mg/kg) and 7 doses of fentanyl (blue) and morphine (red). One-way repeated measures ANOVA and Dunnett's multiple comparisons test identified doses of fentanyl and morphine that caused a significant (*P< 0.02) increase in tail withdrawal latency compared to the respective control (0 mg/kg).

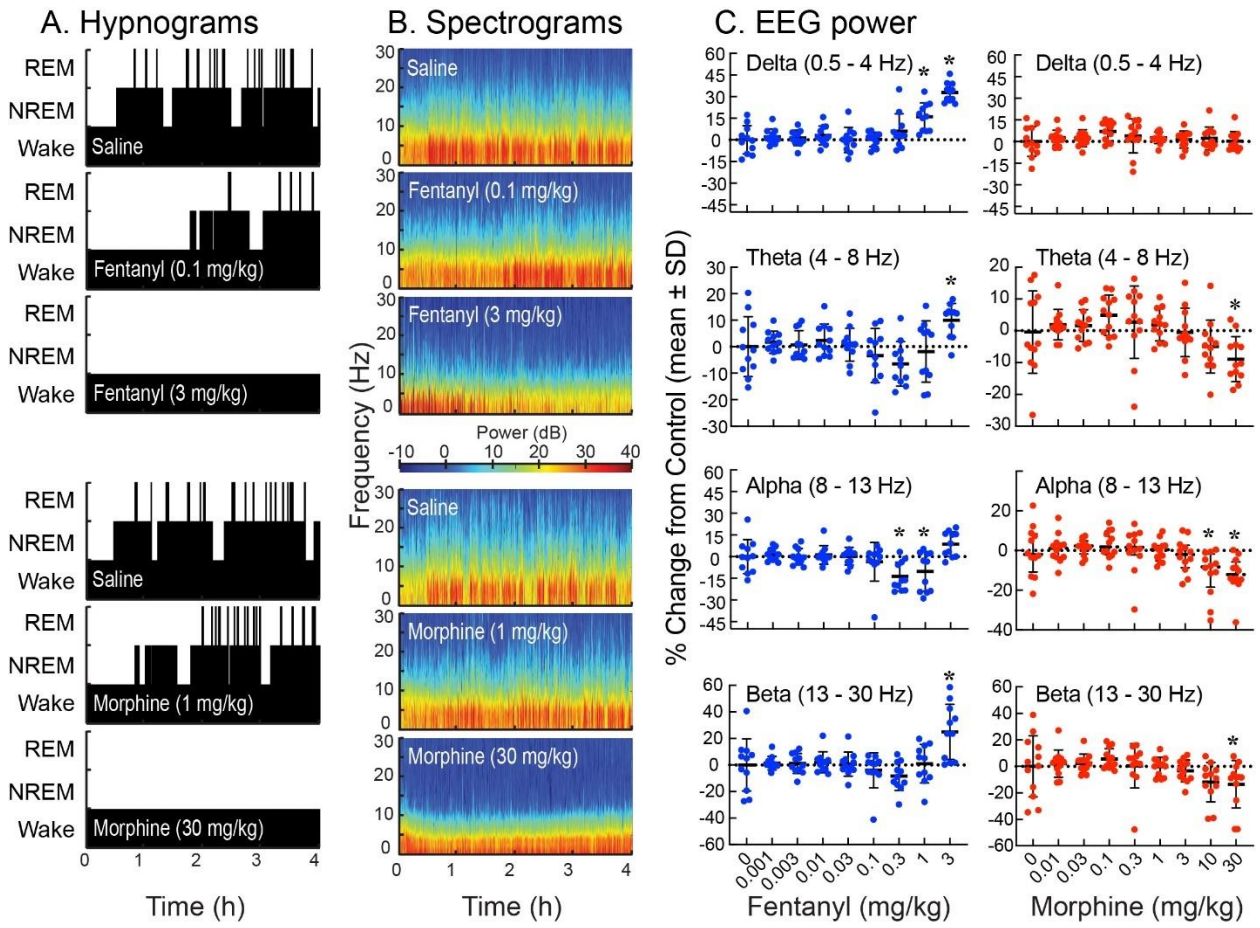
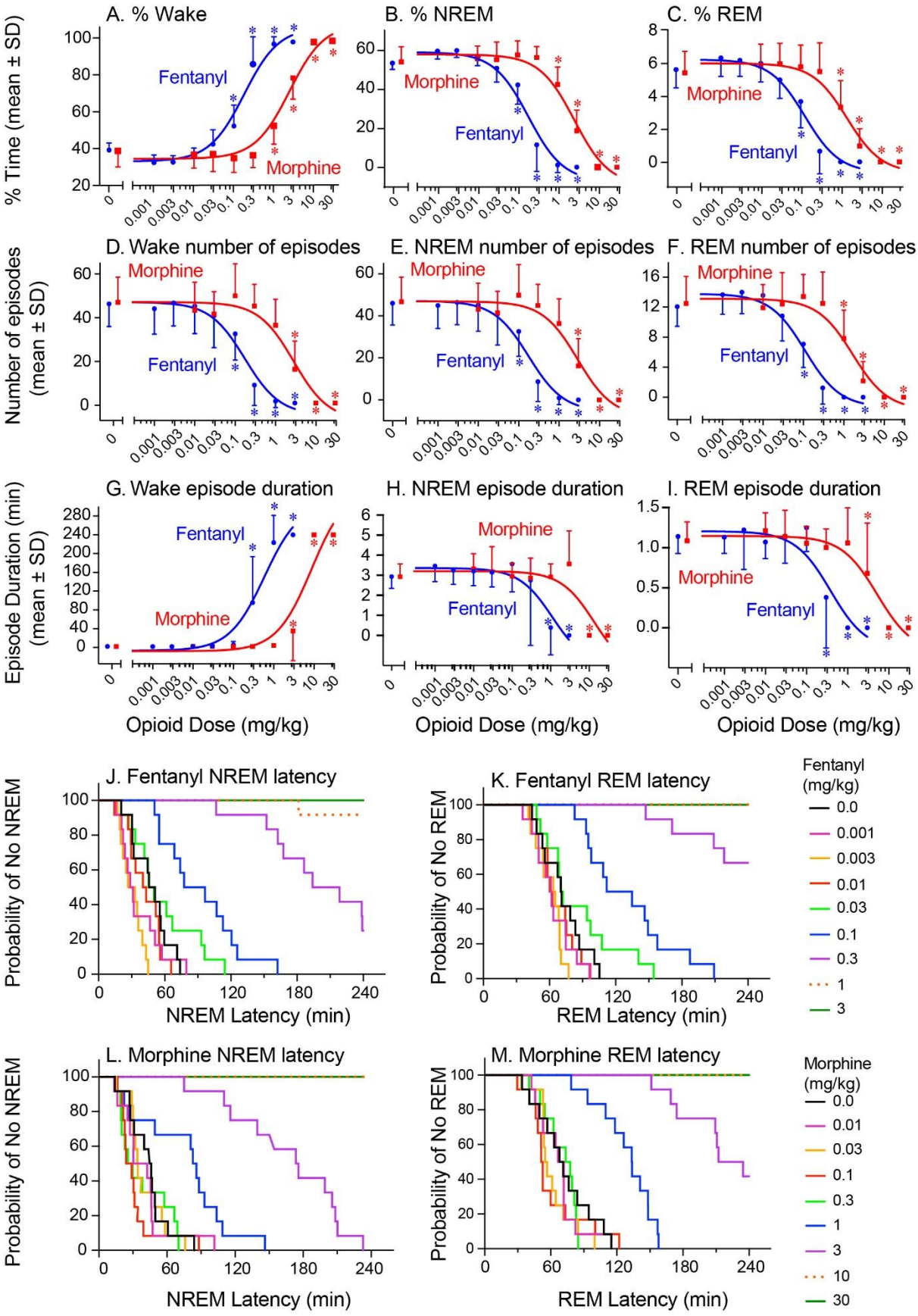


Figure 3.2 Fentanyl and morphine differentially change EEG power during wakefulness.

(A) Representative time course plots of wakefulness (Wake, lowest height black bars), non-rapid eye movement sleep (NREM, intermediate height black bars), and rapid eye movement sleep (REM, highest black bars) from the same mouse during 4 h of recording after receiving injections of saline (vehicle control), two doses (mg/kg) of fentanyl, and two doses of morphine. The mouse was awake for at least 30 min after receiving an injection. The low dose of each opioid decreased both states of sleep, and the highest dose of each opioid eliminated sleep. (**B**) EEG data from the recordings in part A were plotted as multitapered spectrograms to reveal opioid-induced changes in EEG power (decibels, dB) at frequencies ranging from 0 to 30 Hz across the 4-h recoding period. Spectrograms help visualize dose-dependent and differential effects of the two opioids on EEG power. (**C**) EEG power during wakefulness was averaged over the first 30 min post-injection and plotted as percent change from saline (0 mg/kg) for 8 doses of fentanyl (blue, n=11 mice) and 8 doses of morphine (red, n=12 mice). Significant differences from control (*P < 0.05) were identified by repeated measures ANOVA and Dunnett's multiple comparisons test.

Figure 3.3. Dose-dependent effects of opioids on sleep and wakefulness.
All dependent measures were obtained from 12 mice after administration of saline (0 mg/kg) and eight doses of fentanyl (blue) and morphine (red). Dose-response curves (**A-I**) show mean ± SD. Repeated measures ANOVA and Dunnett's multiple comparisons test identified significant differences (*$P < 0.05$) from saline control (0 mg/kg). Survival curves (**J-M**) plot the amount of time post-injection (min) until each mouse entered its first NREM or REM sleep episode. There is one curve per dose, and the key at right shows the color code for each dose.


A. % Wake
B. % NREM
C. % REM
% Time (mean ± SD)
Fentanyl
Morphine
D. Wake number of episodes
E. NREM number of episodes
F. REM number of episodes
Number of episodes (mean ± SD)
G. Wake episode duration
H. NREM episode duration
I. REM episode duration
Episode Duration (min) (mean ± SD)
Opioid Dose (mg/kg)
J. Fentanyl NREM latency
K. Fentanyl REM latency
Probability of No NREM
Probability of No REM
NREM Latency (min)
REM Latency (min)
Fentanyl (mg/kg)
0.0
0.001
0.003
0.01
0.03
0.1
0.3
1
3
L. Morphine NREM latency
M. Morphine REM latency
Morphine (mg/kg)
0.0
0.01
0.03
0.1
0.3
1
3
10
30

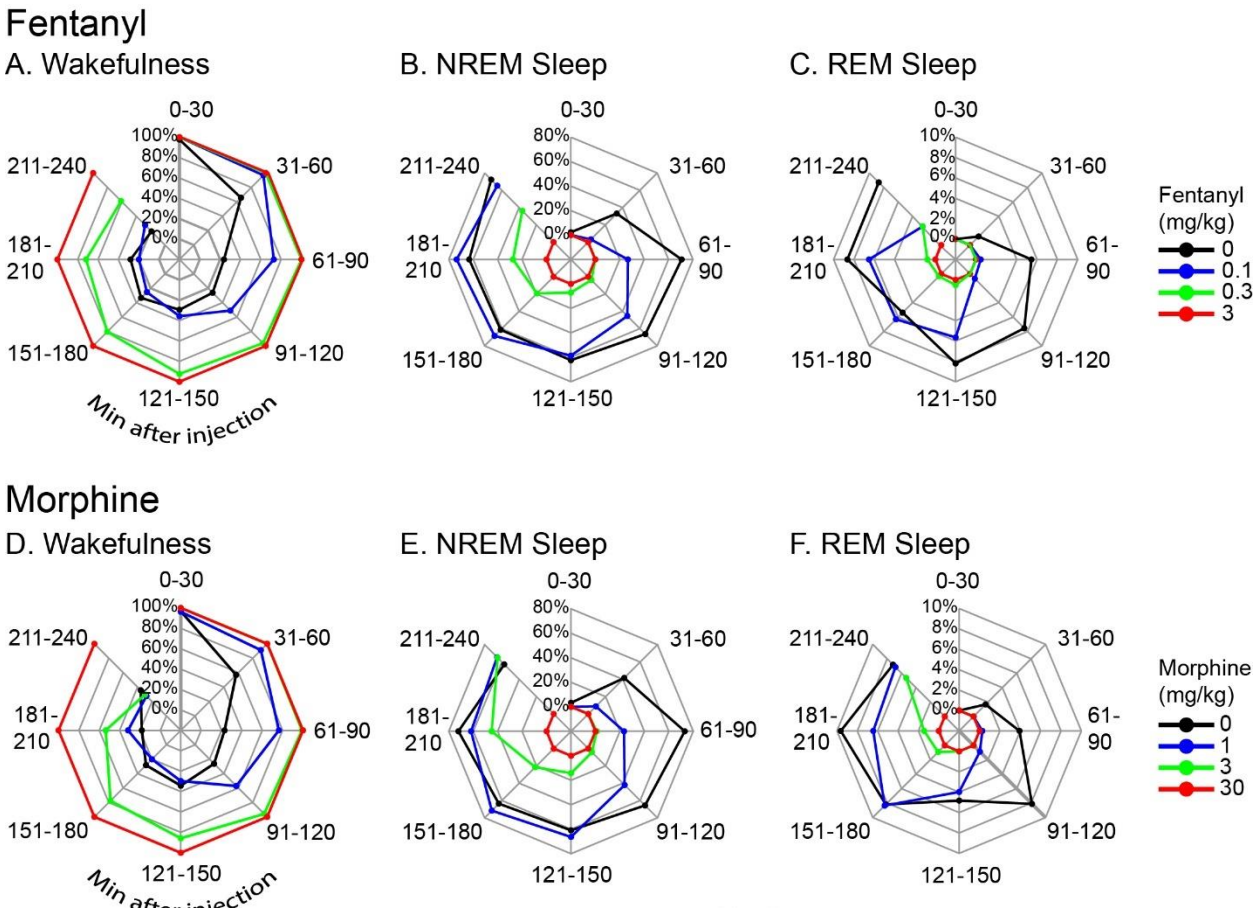


Figure 3.4. Dose-dependent time course of opioid effects on amount of wakefulness, NREM sleep, and REM sleep.
Radar plots show the mean amount of time (min) every 30 min during the 4-h recording period after injection of fentanyl (top row) and morphine (bottom row) that mice (n=12) spent in wakefulness (**A & D**), NREM sleep (**B & E**), and REM sleep (**C & F**). Around the circumference of each radar plot the 8 segments demarcate the 4-h recording period divided into 30 min bins representing time (min) after injection. The key at right identifies the opioid dose. 0 mg/kg indicates saline (vehicle control).

Figure 3.5. Mediation analysis of opioid effects on motor activity, subcutaneous body temperature (Tsc), and wakefulness in 12 mice.
Repeated measures ANOVA and Dunnett's multiple comparisons test identified doses of fentanyl (**A**) and morphine (**D**) that caused a significant (*****$P < 0.05$) change from control (0 mg/kg). Mediation analyses identified the contributions of opioid-induced changes in motor activity (**B, C**) and Tsc (**E, F**) to opioid-induced increases in wakefulness. (**A**) plots the natural logarithm (ln) of activity counts in arbitrary units (a.u.) during wakefulness and (**D**) graphs Tsc during wakefulness in degrees Celsius (^{o}C). Data were averaged across the 4-h recording period after administration of saline and 8 doses of fentanyl (blue) and morphine (red). (**B, C**) Diagrams of the model used to estimate the mediation effect of opioid-induced changes in motor activity on opioid-induced increases in wakefulness. The blue and red arrows indicate causal paths for (**a**) the effect of opioid dose on motor activity, (**b**) the effect of motor activity on % Wake, and (**c'**) the effect of opioid dose on % Wake with motor activity considered. Significant opioid doses (mg/kg) with their respective P values are listed above each of the three arrows (**a, b, c'**) for effects of (**B**) fentanyl dose and (**C**) morphine dose. (**E, F)** Diagrams of the model used to estimate the mediation effect of opioid-induced changes in Tsc on opioid-induced increases in wakefulness. The arrows indicate causal paths for (**a**) the effect of opioid dose on Tsc, (**b**) the effect of Tsc on % Wake, and (**c'**) the effect of opioid dose on % Wake with Tsc considered. Significant opioid doses (mg/kg) with their respective P values are listed above each of the three arrows (**a, b, c'**) for effects of (**E**) fentanyl dose and (**F**) morphine dose. Mediation model diagrams were modified from (Vuorre & Bolger, 2018).

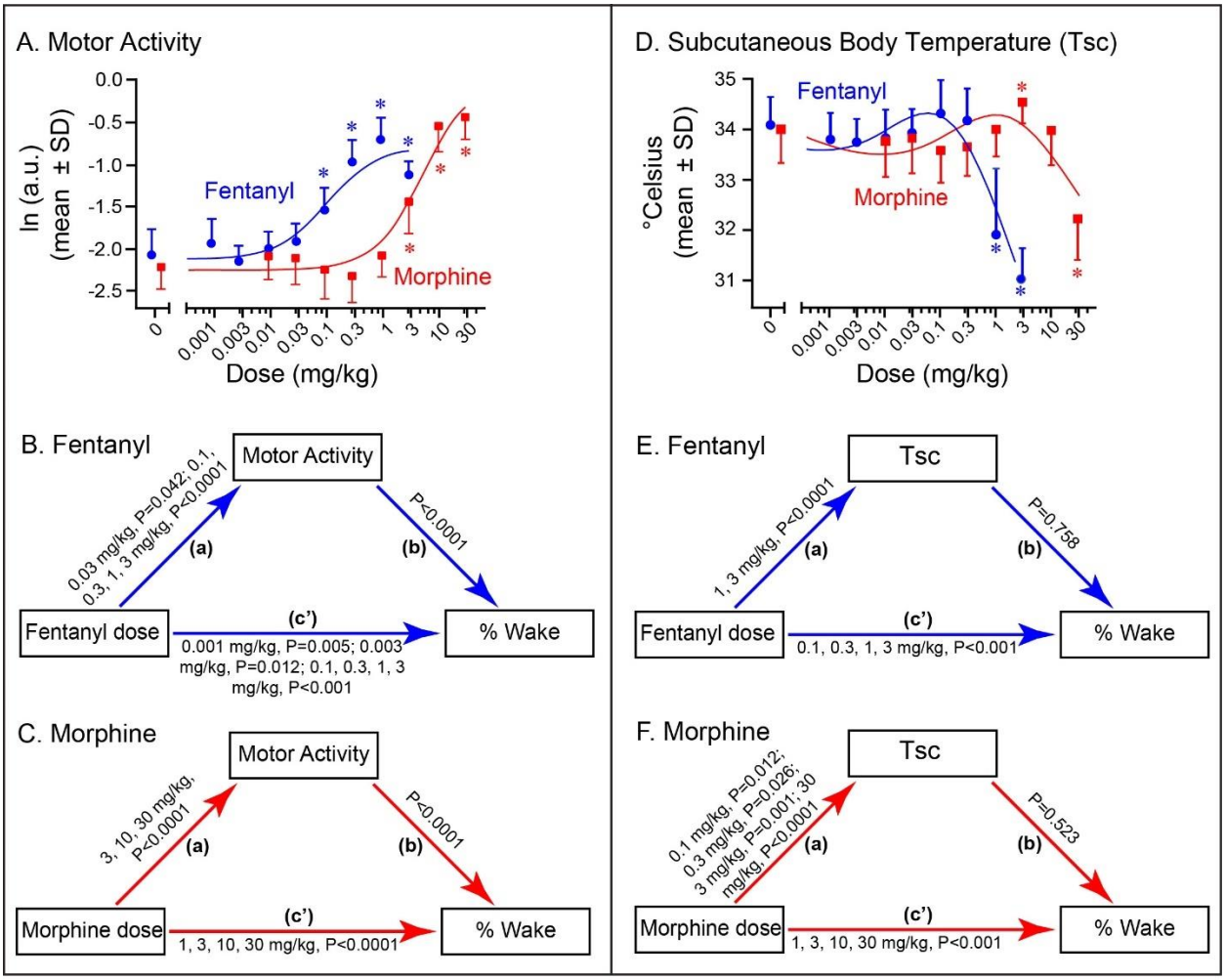

A. Motor Activity
ln (a.u.) (mean ± SD)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Fentanyl
Morphine
0
0.001
0.003
0.01
0.03
0.1
0.3
1
3
10
30
Dose (mg/kg)
B. Fentanyl
Motor Activity
0.03 mg/kg, P=0.042; 0.1, 0.3, 1, 3 mg/kg, P<0.0001
(a)
P<0.0001
(b)
Fentanyl dose
(c')
0.001 mg/kg, P=0.005; 0.003 mg/kg, P=0.012; 0.1, 0.3, 1, 3 mg/kg, P<0.001
% Wake
C. Morphine
Motor Activity
3, 10, 30 mg/kg, P<0.0001
(a)
P<0.0001
(b)
Morphine dose
(c')
1, 3, 10, 30 mg/kg, P<0.0001
% Wake
D. Subcutaneous Body Temperature (Tsc)
°Celsius (mean ± SD)
35
34
33
32
31
Fentanyl
Morphine
0
0.001
0.003
0.01
0.03
0.1
0.3
1
3
10
30
Dose (mg/kg)
E. Fentanyl
Tsc
1, 3 mg/kg, P<0.0001
(a)
P=0.758
(b)
Fentanyl dose
(c')
0.1, 0.3, 1, 3 mg/kg, P<0.001
% Wake
F. Morphine
Tsc
0.1 mg/kg, P=0.012; 0.3 mg/kg, P=0.026; 3 mg/kg, P=0.001; 30 mg/kg, P<0.0001
(a)
P=0.523
(b)
Morphine dose
(c')
1, 3, 10, 30 mg/kg, P<0.001
% Wake

Table 3.1. Number of State Transitions

Treatment	Number of State Transitions (mean ±SD)			
Opioid and Dose (mg/kg)	Wake to NREM	NREM to REM	REM to Wake	NREM to Wake
Fentanyl				
0	46.0 ± 10.4	12.1 ± 2.64	12.0 ± 2.80	33.3 ± 9.20
0.1	32.6 ± 11.9 *	7.08 ± 3.12 *	7.00 ± 3.28 *	24.8 ± 9.44 *
0.3	8.75 ± 9.43 *	1.08 ± 1.73 *	1.08 ± 1.73 *	7.17 ± 8.23 *
3	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *
Morphine				
0	46.1 ± 12.7	12.6 ± 3.45	12.6 ± 3.45	32.9 ± 11.8
1	36.3 ± 11.8	7.83 ± 3.79 *	7.83 ± 3.79	27.7 ± 10.0 *
3	16.2 ± 12.9 *	2.17 ± 2.59 *	2.08 ± 2.39 *	13.3 ± 11.1 *
30	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *

Table 1 reports the number of transitions (mean ± SD) between states of wakefulness (Wake), rapid eye movement sleep (REM), and non-REM sleep (NREM) quantified for 4 h after mice (n=12) received saline (vehicle control, 0 mg/kg), the lowest antinociceptive doses, the next highest doses, and the highest doses tested of fentanyl (0.1, 0.3, 3 mg/kg) and morphine (0, 1, 3, 30 mg/kg). Data were analyzed using repeated measures one-way ANOVA with multiple comparisons to saline (0 mg/kg) provided by Dunnett's (*$P < 0.01$).

Chapter 4 : Discussion

The findings from research comprising this dissertation are discussed in Chapter 2 (page 51) and Chapter 3 (page 116). The discussion sections in those chapters review the present results relative to the published literature. This section focuses on the points not already discussed.

Dissociation between motor activity and body temperature

The endogenous opioid system is key to the regulation of many physiological functions such as body temperature, motor activity, and feeding behavior (Kozak et al., 1995). Motor activity is a well-known regulator of body temperature, and in healthy animals under normal physiological circumstances body temperature increases during exercise. Temperature regulatory systems then set a new balance between heat production and heat dissipation (Gordon & Yang, 1997; Nadel, 1983). In contrast to normal physiological regulation, the results from this dissertation research revealed that during increased motor activity caused by high-dose opioids, B6 mice showed a decrease in body temperature (**Figure 3.5**, pages 145-146).

Circadian rhythms exist in many biological processes, are intrinsically generated, and enable organisms to anticipate environmental changes. By anticipating these shifts, circadian rhythms also prime physiological systems for optimal functionality, thereby ensuring overall well-being and survival (Vitaterna et al., 2001). Motor activity, body temperature, and sleep are regulated, in part, by circadian rhythms (Bergum, Berezin, Dooley, et al., 2022). Circadian rhythms are synchronized by the suprachiasmatic nuclei (SCN) of the hypothalamus via photic input from the retina (Bergum, Berezin, & Vigh, 2022; Bumgarner et al., 2023). Although motor activity and body temperature rhythms are closely synchronized, previous research in small mammals suggests that the body temperature rhythm is not a byproduct of the activity rhythm (Refinetti, 1999). Data in rats suggest that body temperature can change independent of motor activity by

modulation of skin blood flow and behaviors such as feeding (Gordon & Yang, 1997). Opioids have been shown to alter circadian rhythm regulation by modifying the photic responsiveness in the SCN (Vansteensel et al., 2005) and by altering retinal input by forming opioid deposits in the mammalian retina (Bergum, Berezin, Dooley, et al., 2022). Circadian rhythms such as the motor activity rhythm must adapt to the environmental changes of the photoperiod (Benstaali et al., 2001). However, when the input of the photoperiod or feedback loop is altered, the rhythms desynchronize. I speculate that by altering the circadian rhythm, opioids may differentially modulate body temperature and motor activity rhythms, leading to the dissociation observed between these two physiological processes.

Translational relevance of these results has to be taken with caution because opioid effects on motor activity and body temperature are at least partially species specific. Opioids increase motor activity in rodents (Gaulden et al., 2021; Haouzi et al., 2021) and non-human primates (Weed & Hienz, 2006) but cause a state of stupor and drowsiness in humans (Benyamin et al., 2008; Paul et al., 2021). Opioid effects on body temperature depend on both the environment (Sadowski et al., 1996) and the specific opioid administered (Baker & Meert, 2002). Opioids tend to induce hypothermia in rodents (Rawls & Benamar, 2011; Rosow et al., 1980) and humans (Ikeda et al., 1997) whereas in non-human primates, opioids raise body temperature (Weed & Hienz, 2006). In summary, these adverse effects of opioids result from the interactions caused by opioid receptor activation across multiple physiological systems.

A comparative medicine approach to species-specific opioid dosing

Dosing requirements of opioids for pain management in human medicine is facilitated by a complex series of calculations referred to as morphine milligram equivalents (MME). These

calculations use morphine as the gold standard for determining equianalgesic doses of various opioids used in human clinical settings. MME calculations help clinicians select appropriate dosing when switching from one opioid agent or route of administration to another. MME conversions are also commonly used to prevent toxicity (Bhatnagar & Pruskowski, 2022) while managing a patient's pain. Clinicians are also assisted in individualizing doses of various opioids based on type of pain, clinical history, and patient-specific variables (e.g., age, sex, health condition, etc.). Due to the variety of factors that need to be considered, MME calculations are approximations and are intended solely as guides. In fact, a recent study identified that there is no standardized or universal method for calculating daily morphine equivalence (Nguyen et al., 2022).

Although there is some interest in calculating MMEs for non-human animals, at present it has not been possible to translate the assumptions from human medicine to non-human animals, including the B6 mice used in the present studies. This is due, in part, to species-specific pharmacokinetics, pharmacodynamics, routes of administration, and physiology (Bhatnagar & Pruskowski, 2022; Reagan-Shaw et al., 2008). Each species metabolizes drugs slightly differently and doses are adjusted according to body surface area and body weight. Since body composition varies across species, dose translations between species are better performed using body surface area rather than body weight. Based on significant interspecies differences in pharmacokinetics, pharmacodynamics, and body surface area, converting mouse doses into MMEs remains, at best, an approximation. Therefore, it is likely that applying human MME calculations to guide dosing for research studies using mice would yield inaccurate results. The eventual development of MME-like guidelines for laboratory animals and for veterinary

medicine will be based on empirically derived measures such as the dose-response curves and time course data reported in this dissertation. A key advantage of the empirical approach is that after administering 390 doses of fentanyl and morphine, no mice in this study experienced or died from an overdose. Although the use of MME calculations is desired to achieve better translational significance with humans, due to the numerous factors that influence MME conversions and the differences between species, this study opted to present results based solely on mouse-derived doses. Doses were selected, in part, based on guidelines provided by the university IACUC. Notably, all the doses of fentanyl and morphine that significantly disrupted sleep, EEG power, motor activity, and body temperature correspond to antinociceptive doses in male B6 mice.

Concluding Remarks

The findings reported in this dissertation advance understanding of opioid-induced disruption of traits (motor activity, subcutaneous body temperature, and EEG power) as they define neurobehavioral states (wakefulness, NREM sleep, and REM sleep). The purpose of this final section is to provide a higher-level, integrative perspective on the novelty and significance of the present findings as well as future directions.

As a veterinarian, my intensive training in comparative physiology, pharmacology, and pathology stimulated my interest in the complex interactions among different physiological systems in different species. The present studies of B6 mice were reinforced by a vast literature showing homologies between mice and humans in opioid-induced alterations across physiological systems. The present dissertation contains no measures of addiction, but there is good evidence that sleep disruption contributes to all phases of the addiction cycle: onset,

maintenance, and relapse (Roehrs et al., 2021). My interests led me to quantify, in B6 mice, the dose-dependent effects of systemically administered fentanyl and morphine on electrographically defined states of sleep and wakefulness and on EEG power, motor activity, and body temperature. The data are limited to male B6 mice and the result encourage future studies in female mice and obese mice, which are known to have differential responses to opioids.

Four points illustrate the potential clinical relevance of the present preclinical data. The first point concerns the need for opioid research that includes dose-response studies. The safe and effective use of opioids for pain management requires a nuanced understanding that the dose-dependent effects of opioids vary as a function of species and subject variables such as sex, age, body mass, and coexisting injury or disease. As I was approaching the end of these studies it was gratifying to read in the neuroscience literature an editorial emphasizing the need for dose-response studies of opioids (Burgraff, 2024). Based on my review of currently published data, the present results provide, for the first time in B6 mice, 4-log unit dose-response data for the effects of fentanyl and morphine on sleep/wake states and simultaneously acquired measures of EEG power, motor activity, and subcutaneous body temperature.

A second example of the potential clinical relevance of the data shown in this dissertation concerns the persisting challenge for clinical care across species to provide effective pain management without also disrupting states of sleep and wakefulness. In addition, the present results provide the first dose-response data in B6 mice showing that the lowest antinociceptive doses of fentanyl and morphine also disrupt sleep. Thus, data from this dissertation encourage future studies designed to provide data-based protocols for adjunctive medications that diminish nociception without causing sleep disruption.

Quantification of opioid-induced disruption of EEG power is a third area of potential clinical relevance for efforts to enhance pain management without depressing neurocognitive function. The present EEG data from healthy B6 mice encourage future studies of opioid effects on EEG recorded from mice with validated models of acute and chronic pain.

Finally, the present study was encouraged by a question I received while presenting my initial preliminary data (Zebadúa Unzaga et al., 2021) on the extent to which opioid-induced sleep disruption is due to enhancement of motor activity. Addressing this question required simultaneous measurement of sleep/wake states, motor behavior and, as reviewed in Chapter 3, body temperature. This question was addressed using mediation analysis, a form of structural equation modeling. The results present the first quantitative data showing that opioid-induced sleep disruption is partially mediated by motor activity but not by body temperature. These four novel findings share an integrative approach common to medicine and systems physiology: complex interactions and a lack of independence from the level of the cell to the level of the organ systems.

References

Baker, A. K., & Meert, T. F. (2002). Functional effects of systemically administered agonists and antagonists of μ, δ, and κ opioid receptor subtypes on body temperature in mice. *The Journal of Pharmacology and Experimental Therapeutics*, *302*(3): 1253-1264. https://doi.org/10.1124/jpet.102.037655

Benstaali, C., Mailloux, A., Bogdan, A., Auzéby, A., & Touitou, Y. (2001). Circadian rhythms of body temperature and motor activity in rodents: Their relationships with the light-dark cycle. *Life Sciences*, *68*(24): 2645-2656. https://doi.org/10.1016/S0024-3205(01)01081-5

Benyamin, R., Trescot, A. M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., Glaser, S. E., & Vallejo, R. (2008). Opioid complications and side effects. *Pain Physician*, *11*(2 Suppl): S105-120. https://doi.org/10.36076/ppj.2008/11/S105

Bergum, N., Berezin, C.-T., Dooley, G., & Vigh, J. (2022). Morphine accumulates in the retina following chronic systemic administration. *Pharmaceuticals*, *15*(5).

Bergum, N., Berezin, C.-T., & Vigh, J. (2022). A retinal contribution to opioid-induced sleep disorders? [Mini Review]. *Frontiers in Neuroscience*, *16*. https://doi.org/10.3389/fnins.2022.981939

Bhatnagar, M., & Pruskowski, J. (2022). *Opioid equivalency* (T. I. (FL), Ed.). StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC. https://www.ncbi.nlm.nih.gov/books/NBK535402/

Bumgarner, J. R., McCray, E. W., & Nelson, R. J. (2023). The disruptive relationship among circadian rhythms, pain, and opioids [Review]. *Frontiers in Neuroscience*, *17*. https://doi.org/10.3389/fnins.2023.1109480

Burgraff, N. J. (2024). The complexity of fentanyl's impact on brain and peripheral oxygenation. *Journal of Neurophysiology*, *132*(2): 319-321. https://doi.org/10.1152/jn.00256.2024

Gaulden, A. D., Burson, N., Sadik, N., Ghosh, I., Khan, S. J., Brummelte, S., Kallakuri, S., & Perrine, S. A. (2021). Effects of fentanyl on acute locomotor activity, behavioral sensitization, and contextual reward in female and male rats. *Drug and Alcohol Dependence*, *229*: 109101. https://doi.org/10.1016/j.drugalcdep.2021.109101

Gordon, C. J., & Yang, Y. (1997). Contribution of spontaneous motor activity to the 24 hour control of body temperature in male and female rats. *Journal of Thermal Biology*, *22*(1): 59-68. https://doi.org/10.1016/S0306-4565(96)00035-6

Haouzi, P., McCann, M., & Tubbs, N. (2021). Respiratory effects of low and high doses of fentanyl in control and β-arrestin 2-deficient mice. *Journal of Neurophysiology*, *125*(4): 1396-1407. https://doi.org/10.1152/jn.00711.2020

Ikeda, T., Kurz, A., Sessler, D. I., Go, J., Kurz, M., Belani, K., Larson, M., Bjorksten, A. R., Dechert, M., & Christensen, R. (1997). The effect of opioids on thermoregulatory responses in humans and the special antishivering action of meperidine. *Annals of the New York Academy of Sciences*, *813*(1): 792-798. https://doi.org/10.1111/j.1749-6632.1997.tb51783.x

Kozak, W., Conn, C. A., & Kluger, M. J. (1995). Body temperature, motor activity, and feeding behavior of mice treated with β-chlornaltrexamine. *Physiology & Behavior*, *58*(2): 353-362. https://doi.org/10.1016/0031-9384(95)00067-S

Nadel, E. R. (1983). Factors affecting the regulation of body temperature during exercise. *Journal of Thermal Biology*, *8*(1): 165-169. https://doi.org/10.1016/0306-4565(83)90097-9

Nguyen, C. T., McPherson, M. L., & Walker, K. (2022). Rs8: Not so surprising- inconstancy of oral morphine equivalents (ome) calculation practices. *Journal of pain & palliative care pharmacotherapy*, *35*(4): 232-232. https://doi.org/10.1080/15360288.2021.1981675

Paul, A. K., Smith, C. M., Rahmatullah, M., Nissapatorn, V., Wilairatana, P., Spetea, M., Gueven, N., & Dietis, N. (2021). Opioid analgesia and opioid-induced adverse effects: A review. *Pharmaceuticals*, *14*(11): 1091. https://doi.org/10.3390/ph14111091

Rawls, S. M., & Benamar, K. (2011). Effects of opioids, cannabinoids, and vanilloids on body temperature. *FBS*, *3*(3): 822-845. https://doi.org/10.2741/190

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *The FASEB Journal*, *22*(3): 659-661. https://doi.org/10.1096/fj.07-9574LSF

Refinetti, R. (1999). Relationship between the daily rhythms of locomotor activity and body temperature in eight mammalian species. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *277*(5): R1493-R1500. https://doi.org/10.1152/ajpregu.1999.277.5.R1493

Roehrs, T., Sibai, M., & Roth, T. (2021). Sleep and alertness disturbance and substance use disorders: A bi-directional relation. *Pharmacology Biochemistry and Behavior*, *203*(173153): 1-8. https://doi.org/10.1016/j.pbb.2021.173153

Rosow, C. E., Miller, J. M., Pelikan, E. W., & Cochin, J. (1980). Opiates and thermoregulation in mice. I. Agonists. *Journal of Pharmacology and Experimental Therapeutics*, *213*(2): 273-283. https://jpet.aspetjournals.org/content/213/2/273

Sadowski, B., Sas, R., Swiderski, T., & Trzasko, M. (1996). Differentiation of opioid and nonopioid pain inhibitory mechanisms, and of thermoregulation in mice selectively bred

for high and low swim stress-induced analgesia. *Analgesia, 2*(4): 211-218. https://www.ingentaconnect.com/contentone/cog/ana/1996/00000002/00000004/art00005

Vansteensel, M. J., Magnone, M. C., Van Oosterhout, F., Baeriswyl, S., Albrecht, U., Albus, H., Dahan, A., & Meijer, J. H. (2005). The opioid fentanyl affects light input, electrical activity and per gene expression in the hamster suprachiasmatic nuclei. *European Journal of Neuroscience, 21*(11): 2958-2966. https://doi.org/10.1111/j.1460-9568.2005.04131.x

Vitaterna, M. H., Takahashi, J. S., & Turek, F. W. (2001). Overview of circadian rhythms. *Alcohol Res Health, 25*(2): 85-93.

Weed, M. R., & Hienz, R. D. (2006). Effects of morphine on circadian rhythms of motor activity and body temperature in pig-tailed macaques. *Pharmacology Biochemistry and Behavior, 84*(3): 487-496. https://doi.org/10.1016/j.pbb.2006.06.012

Zebadúa Unzaga, D., Bustamante, C., Thibert, M., & Baghdoyan, H. A. (2021). Fentanyl and morphine cause dose-dependent sleep disruption in c57bl/6j (b6) mice. *The FASEB Journal, 35*. https://doi.org/10.1096/fasebj.2021.35.S1.01706

Vita

Born and raised in Mexico City, Diana Zebadúa Unzaga studied Veterinary Medicine and Zootechny at the Autonomous National University of Mexico. As a fourth-year veterinary student, Diana discovered her passion for internal medicine and the complexities of physiological systems. This led her to pursue an internship in large animal medicine in the College of Veterinary Medicine in Guelph, Canada, followed by a residency in large animal internal medicine at the College of Veterinary Medicine, University of Tennessee Knoxville. During her residency she developed interests in teaching and research, which inspired her to enroll in the Doctor of Philosophy degree program in Experimental Psychology. Under the mentorship of Dr. Helen A. Baghdoyan and Dr. Ralph Lydic, Diana's dissertation research focused on the systems physiology perspective of opioid-induced sleep disruption.