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using confocal imaging. The project also included a clinical component in partnership with Newton-Wellesley Hospital, in which I worked with a surgeon to consent patients undergoing hysterectomies to donate their waste endometrial tissue for our study. This was an eye-opening project because it allowed me to clearly see the connection between the research I was doing at the bench and its impacts on human health. I also submitted a proposal on this work and was funded by the MIT research office.

1.Smith PC, Meyer AS, Lauffenburger D. *Investigation of an Array of Axl-Mediated Growth Factor Dependent Migratory Effects.* Funded proposal submission to the MIT UROP office, 2011.

Graduate Rotations: During my rotation in the lab of Dr. Anthony Kim, I presented a poster and received a first-place cash award for my poster on the development of nanoparticles for glioblastoma treatment, as well as co-authored a book chapter with the lab of Dr. J. Marc Simard on the use of robotics in neurosurgery.

1. Stokum JA, Kurland D, Smith PC, Simard JM. Chapter in *Encyclopedia of Medical Robotics*, editor-in-chief: Jaydev P. Desai. 2017.

2. Smith PC, Kim AJ. *Optimization of Nanoparticles for Effective Drug Transport in the Treatment of Glioblastoma.* Poster presentation, Medical Student Research Day, University of Maryland, Baltimore. First-place cash award received.

Graduate Thesis Work: I am currently a graduate student in the lab of Dr. Jessica Mong at the University of Maryland, Baltimore. My current project focuses on leveraging my knowledge of microenvironmental and applies them to the neuroscience field to explore

the interaction between the endocrine system and the biological basis of sleep. I pursued three different rotations, including a very productive rotation with Dr. Kim, but found Dr. Mong's mentorship and neuroscience focus to be the best mentoring fit for my style. Furthermore, I have enjoyed the chance to apply my diverse background to sleep, an area with broad multi-systemic effects on human health. I have prepared scientific manuscripts on my work and a review paper on neuroendocrine control of sleep. I have presented a scientific poster at the Neuroscience 2018 and 2019 conferences and an oral presentation at the 34th annual national MD/PhD student conference. I have also received a funded NRSA F30 Grant from the National Heart, Lung, and Blood Institute.

1. Smith PC, Viechweg SS, Buck S, Pocivavsek A, Mong JA. *Estrogen Modulates Adenosinergic Sleep Pressure.* Poster Presentation, Neuroscience 2018, San Diego CA.

2. Smith PC, Phillips DJ, Pocivavsek A, Viechweg SS, Mong JA. *Estrogen Modulates Adenosinergic Sleep Pressure.* Poster Presentation, Neuroscience 2019, Chicago, IL

3. Smith PC, Phillips DJ, Pocivavsek A, Viechweg SS, Mong JA. *Estrogen Modulates Adenosinergic Sleep Pressure.* Oral Presentation, 34th MD/PhD Student Conference, Copper Mountain, CO.

4. Smith PC, Mong JA. *"Neuroendocrine Control of Sleep."* Current Topics in Behavioral Neurosciences, vol. 43, 2019, pp. 353–78. PubMed, doi:10.1007/7854_2019_107.

Research Support Awards

1. Smith PC, Mong JA. *Mechanisms of Estrogenic Modulation of Adenosinergic Sleep Pressure*. NIH F30 #1HL145901, awarded February 2019

Abstract

To further our understanding of how gonadal steroids impact sleep biology, we sought to address the mechanism by which proestrus levels of cycling ovarian steroids, particularly estradiol (E2), suppress sleep in female rats. We showed that steroid replacement of proestrus levels of E2 to ovariectomized female rats, suppressed sleep to similar levels as those reported by endogenous ovarian hormones. We further showed that this suppression is due to the high levels of E2 alone, and that progesterone did not have a significant impact on sleep behavior. We found that E2 action within the Median Preoptic Nucleus (MnPN), which contains estrogen receptors (ERs), is necessary for this effect; antagonism of ERs in the MnPN attenuated the E2-mediated suppression of both non-Rapid Eye Movement (NREM) and Rapid Eye Movement (REM) sleep. Finally, we found E2 action at the MnPN is also sufficient for sleep suppression, as direct infusion of E2 into the MnPN suppressed sleep. Based on our findings, we predict proestrus levels of E2 alone, acting at the MnPN, mediate sex-hormone driven suppression of sleep in female rats.

Furthermore, our findings have demonstrated that E2 has different effects on sleep time and slow wave activity (NREM-SWA), a measure of the homeostatic efficiency of sleep, under conditions of normal sleep and sleep deprivation. E2 serves to decrease NREM-SWA, but not sleep time, in the light phase under normal sleep, while in recovery, E2 decreases sleep time but not NREM-SWA following deprivation. We further found that E2 increased levels of extracellular adenosine, a measure of homeostatic sleep need, under both normal and deprivation sleep conditions. To resolve the discrepancy between the decrease in behavioral markers associated with homeostatic sleep need and increase in levels of adenosine, we employed an agonist of the A2A receptor to stimulate the

adenosinergic sleep pressure system. These studies show that E2 blunts the ability of the A2A agonist to drive sleep behavior, showing an interposition of E2 into the adenosinergic sleep pressure system. Overall, these experiments show an interaction between E2 and adenosinergic homeostatic sleep pressure at the level of the MnPN.

Estradiol Action at the Median Preoptic Nucleus Modulates Adenosinergic Homeostatic Sleep Pressure

by
Philip Carver Smith III

Dissertation submitted to the Faculty of the Graduate School of the
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List of Abbreviations

5HT	Serotonin
ACH	Acetylcholine
ADA	Adenosine Deaminase
AMP	Adenosine Monophosphate
ASPD	Advanced Sleep Phase Disorder
ATP	Adenosine Triphosphate
AUC	Area under the Curve
BF	Basal Forebrain
CGS	Adenosine 2A Agonist CGS-21680
CPAP	Continuous Positive Airway Pressure
CRH	Corticotropin Releasing Hormone
CSF	Cerebrospinal Fluid
DA	Dopamine
DMSO	Dimethyl Sulfoxide
DSPD	Delayed Sleep Phase Disorder
E1	Estrone
E2	Estradiol

E3	Estriol
EEG	Electroencephalogram
EMG	Electromyogram
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
Fos-ir	Fos Immunoreactivity
GH	Growth Hormone
Hist	Histamine
HPA	Hypothalamic-Pituitary Axis
ICI	ER Antagonist ICI-182-780
ISF	Interstitial Fluid
KPBS	Potassium Phosphate Buffered Saline
LC	Locus Coeruleus
LDT	Laterodorsal Tegmental Nucleus
LH	Lateral Hypothalamus
L-PGDS	Lipocalin-type Prostaglandin D Synthase
MCH	Melanin Concentrating Hormone
MnPN	Median Preoptic Nucleus

NA	Noradrenaline
nCPA	n-Cyclopentyl Adenosine
NREM	Non-Rapid Eye Movement Sleep
ORX	Orexin
OSA	Obstructive Sleep Apnea
OVX	Ovariectomy
P	Progesterone
PB	Parabrachial Nucleus
PFZ	Parafacial Zone
PPT	Pedunculopontine Nucleus
REM	Rapid Eye Movement Sleep
RLS	Restless Legs Syndrome
SAD	Seasonal Affective Disorder
SAH	S-adenosyl Homocysteine
SCN	Suprachiasmatic Nucleus
SNIPP	Sleep Need Index Phosphorylation Prot.
SUM	Supramammilary Nucleus
SWA	Slow Wave Activity

TMN	Tuberomammillary Nucleus
VEH	Vehicle
VLPO	Ventral Lateral Preoptic Nucleus
VPAG	Ventral Periaqueductal Gray Matter
ZT	Zeitgeber Time

I. Background and Introduction

A. Introduction

Sleep is a phenomenon in animal behavior as enigmatic as it is ubiquitous, and one deeply tied to endocrine function. Sleep is generally defined as being characterized by an increased threshold for response to sensory input, a decrease in motor function, and a lack of consciousness and in general terms is comprised of two clearly defined sleep states, non-rapid eye movement and rapid eye movement (NREM and REM, respectively) which are defined in detail below .

Though humans spend on average a third of their lifespan in the sleep state[1] there are still many unanswered questions about the neurochemical basis of sleep and its functions. Extensive interactions have been identified between sleep and the endocrine system, both in endocrine effect on sleep and sleep effects on the endocrine system. Numerous endocrine factors can affect sleep quantity and quality, while studies have shown a profound effect of sleep behavior on overall endocrine function and stability.[2-3] Further investigation of the neurobiological and neuroendocrine basis of sleep could have wide impact on a number of clinical and basic science fields, from treatment of insomnia to exploration of sex-distinct sleep differences to investigation of the pathogenesis of neurodegenerative diseases. In this thesis we summarize the state of basic sleep biology and its connections to the field of neuroendocrine biology, as well as suggest key future directions for the neuroendocrine regulation of sleep that may significantly impact new therapies for sleep disorders in women and men.

1. Sex Differences in Sleep Remain Understudied

Primary sleep disorders are among the most common medical conditions; as many as one in three individuals may have sleep problems with an annual economic impact of over $100 billion. Emerging clinical evidence suggests that chronic insufficient sleep and sleep disorders have severe health consequences for both women and men. It is estimated that as much as 6% of the general population suffers from clinically-diagnosable insomnia, and as many as a third of individuals show some symptoms.[4] While chronic insufficient sleep is a risk factor in both sexes for a variety of psychological, neurological, and neurodegenerative pathology as well as cardiovascular and metabolic dysfunctions, clinical studies reveal that women suffering from sleep disturbances are at greater risk than men for disorders such as depression as well as metabolic and cardiovascular dysfunction.[5] Furthermore, compared to men and boys, women and girls are twice as likely to experience sleep disruptions and insomnia throughout their lifespan.[6] Given risks to well-being, sleep disorders among women are a significant public health concern.

This discrepancy may be due to a multitude of factors, including chromosomal sex differences and psychosocial factors such as anxiety prevalence. However, there is reason to believe the female profile of steroid hormones may play a role. Clinical data suggest the increased risk of sleep disruption in women emerges at puberty and has been associated with fluctuations in ovarian steroids, particularly estrogens. This evidence suggests that gonadal steroids and biological sex are significant risk factors for sleep disruptions,[5] though it is unclear if sex steroids affect sleep in men.[5] Apart from the clinical finding that sex steroids may affect sleep behavior and architecture, the mechanisms underlying a link between steroids and sleep disturbances remain a gap in our knowledge. In particular, despite a growing understanding of sleep regulatory mechanisms, how estrogens influence

the sleep circuitry is poorly understood. To gain a better understanding of sleep disruptions and insomnia, we must first understand how estrogens influence basic sleep mechanisms in females.

Historically, male rodents alone have served as the cornerstone for elucidating the neural circuitries governing sleep. Unfortunately, this has resulted in a significant gap in our understanding of how estrogens modulate these circuits in females. The female rodent offers the opportunity to probe the neurological sleep circuitry to elucidate the mechanisms by which ovarian steroids modulate sleep. In particular, it has been well established that sleep patterns in the female rat are exquisitely sensitive to natural fluctuations in ovarian steroids such as estradiol (E2).[6-8] The highly reproducible effects of E2 on sleep in female rodents provide an informative bioassay to investigate how E2 may be affecting sleep mechanisms. The use of animal models is critical for advancing our understanding of the potential endocrine-sleep nexus. While much is known about the mechanics of sleep, the investigation into ovarian steroid modulation of sleep is in its infancy; it remains unanswered how ovarian steroids, particularly estrogens, are modulating sleep. Gaining a better understanding of how gonadal steroids influence the basic mechanisms of sleep is imperative if we are to understand how a dysregulated neuroendocrine-sleep circuitry system influences the risk for sleep disorders in women. Such understanding is crucial for the development of appropriate therapies informed by the female physiology. Sex differences in sleep may be due to both a sexually differentiated sleep circuitry programmed pre- or neo-natally and steroidal modulation of the sleep circuit activity.[6] Understanding the mechanisms that influence sleep in females may also provide valuable insights for males, leading to tailored therapies that benefit each sex.

2. Diverse Models Exist for Investigation of Endocrine Regulation of Sleep Behavior

As with many investigations of neurobiological behavior phenomena, animal models are a key tool in the sleep field. Rats are the most commonly-used model of sleep behavior, with mice as a secondary rodent organism. Rats are widespread models for sleep studies as the circuitry and neurochemistry of sleep share similarities with humans, and pharmacologic manipulation and EEG measurement of sleep states are both feasible. Use of mice in the sleep field is generally limited to exploration of sleep in the context of specific genetic backgrounds,[5] which are more readily available in mice than rats. A key consideration in the use of models for sleep is the different patterns of sleep between animals. Sleep is generally entrained to the light-dark cycle through the circadian sleep system, but the specific pattern of sleep differs dramatically between species. Under normal conditions, humans are monophasic sleepers, with a single consolidated period of 7-8 hours per day, concentrated in the dark portion of the light/dark cycle. Conversely, however, rodents are polyphasic sleepers, with many periods of sleep and wake throughout the day.[9] While rodents do have some periods of consolidated wake or sleep of an hour or more, they can also experience bouts as short as a few seconds in duration.[10] Additionally, while rodents do preferentially sleep during the light period of the light-dark cycle, they exhibit significant periods of both wake and sleep throughout the entire light-dark cycle. Unlike rodents, the other major animal model system for sleep, drosophila, are largely monophasic sleepers, with a single consolidated period of sleep entrained to the dark portion of the cycle.[11] As a result, drosophila may be a more useful model of some aspects of the circadian system where light-dark dependence and sleep consolidation are key points of experimental investigation.

3. Female Sex Steroid Hormones Have Myriad Physiological Effects

The most prominent female sex steroids include estrogens and progesterone. In particular, E2 is the most physiologically common and potent form of estrogen. E2 is produced predominantly in the ovaries of females, but is also produced in multiple other tissues in both sexes, including fat, brain, and blood vessels.[12] Like other steroids, its ultimate precursor is cholesterol, which is cleaved to pregnenolone; pregnenolone then undergoes several steroidogenesis reactions. The most common immediate precursor of E2 is testosterone, which is converted to E2 through the actions of aromatase.[13] However, E2 can also be produced indirectly through aromatase action on androstenedione, to produce a related estrogen, estrone (E1).[13] E1 can be converted to E2, and vice-versa, through reversible action of 17-Beta hydroxysteroid dehydrogenase.[14] Hydroxylation of E2 can produce the third major estrogen, estriol (E3), which is primarily produced by the placenta during pregnancy.[15] While it is also produced by the liver in small quantities, circulating levels of E3 are nearly undetectable outside of pregnancy.[16] E2 is about 10 times more potent than E1, which itself is about 10 times more potent than E3.[17] E2 is eliminated through multiple pathways, including conjugation to multiple water-soluble forms and conversion to the less-active E1 and E3.[18]

E2 is important for the development of female secondary sexual characteristics and has diverse effects on multiple organ systems, including breast, skin, bones, liver, and blood vessels, as well as the brain.[19] E2 signaling acts primarily through a pair of nuclear steroid estrogen receptors (ERs), Estrogen Receptor Alpha (ERα) and Estrogen Receptor Beta (ERβ).[20] However, five membrane-bound ERs have also been identified: membrane-bound forms of ERα and ERβ, as well as three others, ER-X, GPR30 and Gq-mER.[21] E2

has been shown to have little signaling at other steroid hormone receptors; while it can signal weakly to antagonize the androgen receptor,[22] in practice this phenomenon is not known to have physiologically relevant effects.

Progesterone is the major non-estrogen female sex steroid. Produced from pregnenolone,[23] progesterone also signals through dedicated nuclear and membrane- bound receptors. It has a number of functions for female reproduction, including breast development and uterine implantation preparation. Progesterone has also been shown to have significant neuromodulatory effects, including counteracting addictive pathways through the enhancement of serotonin receptors.[24] Progesterone administration at high doses, 9mg intranasal or 300mg oral, has also been shown to clinically enhance sleep in postmenopausal women.[25-26] This effect may be due to metabolism to allopregnanolone, a progesterone metabolite known to activate the GABA-A receptor, as GABA-A agonists recapitulate this effect of progesterone and GABA-A antagonists block it.[27-28]

4. Sleep Behavior and Circadian Timing Impact Multiple Hormonal Functions

Multiple different hormones have been shown to be impacted by the sleep-wake cycle, though it remains something of an open question what roles the intrinsic circadian timekeeper and sleep behavioral cycle play in governing the differential release. Indeed, there is evidence to suggest different hormones may display different mechanisms of diurnal entrainment vis-à-vis circadian timing, sleep behavior, and intrinsic homeostatic sleep need.[29]

Among the most important hormonal pathways regulated by sleep is the Hypothalamic- Pituitary- Adrenal (HPA) axis. (Fig. 1) The circadian timekeeper of the Suprachiasmatic Nucleus (SCN) sends projections to the hypothalamus, which result in an

oscillating hormone secretion rhythm in line with the diurnal cycle. The SCN also sends direct neuronal projections to the pituitary, further entraining the release of glucocorticoids in a stereotypical diurnal rhythm. In humans, cortisol levels peak in the early morning just after the onset of the wake phase,[30] a phenomenon thought adapted to prepare the body for wake-time activity. This cortisol rhythm is light-entrained, meaning that significant disruptions have been shown in shift workers and others with circadian rhythm disruption.[30-31]

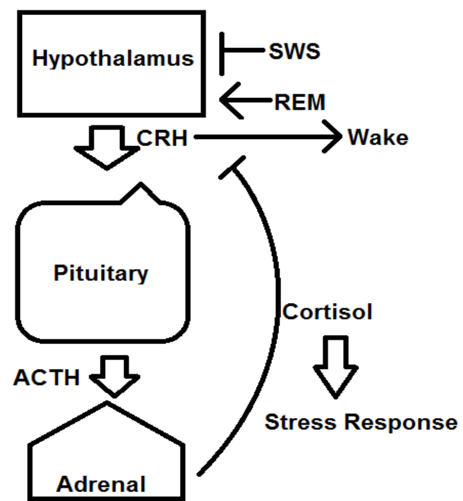


Fig. 1. Schematic of Sleep Impacts on the HPA Axis. Slow Wave Sleep is an inhibitor of the HPA Axis Stress Pathway, while REM sleep stimulates cortisol production. Corticotropin Releasing Hormone (CRH) has been shown to stimulate wake, while exogenous glucocorticoids have been shown to counteract this effect and stimulate sleep by feedback inhibition.

The HPA axis and cortisol play a major role in regulation of the stress response, and sleep has long been shown to have a potent inhibitory influence on this pathway. Sympathetic nervous activity and its downstream effects, including cardiovascular function, display a dependence on sleep state.[32] Slow wave sleep in particular is an inhibitor of HPA axis activity; consequently, cortisol is elevated in the later portions of a sleep bout and during REM sleep.[33] Furthermore, sleep displays a modulation of the adrenal response of cortisol production to the action of adrenocorticotropic hormone; adrenal ACTH sensitivity has also been shown to vary with the diurnal cycle.[34] Further downstream, cortisol rhythms have been shown to affect the immune system; as cortisol is a potent immune and inflammatory suppressor, circadian

disruption has been shown to increase inflammatory cytokines and inflammatory pathologies, including cancers.[34-35]

Insulin is another hormone entrained rhythmically. Insulin, a pancreatic hormone, has a principal function of promoting the absorption of glucose from the blood. However, in experiments of clamping glucose concentration, insulin still rises in the late phase of the sleep cycle,[36] most likely to restrain hepatic glucose production in order to prevent a glycemic peak. This rhythm appears to be SCN-based by studies of dysregulated feeding in SCN-lesioned rats;[37] however, there is also evidence that the pancreatic Beta cells responsible for insulin secretion have their own set of clock transcription factors that may operate independently.[38] Insulin response is also circadian-modulated, with insulin sensitivity in adipose tissue being significantly higher in daytime hours.[39] Sleep deprivation has been shown to be sufficient to induce insulin resistance by multiple pathways.[40]

Similarly, another key hormone for metabolism, growth hormone (GH), which promotes lipolysis and muscle growth, displays cycles entrained to sleep activity.[41] GH is elevated in the earlier portions of a sleep bout, particularly in slow wave sleep, and decreases in later sleep phases.[42] These hormonal changes, as well as similar sleep-dependent fluctuation in the appetite stimulating and suppressing hormones ghrelin[36] and leptin,[30] may in part explain the correlation between sleep disruption and obesity and metabolic syndrome.

Circadian factors have also been shown to have an effect on reproductive hormones. However, studies of the circadian cycle and reproductive-related hormones have been complicated by the fact that the connection appears much stronger in rodents than in humans.[5] For example, in rodents, the surge of luteinizing hormone occurs just before the

onset of the dark phase (and thus mating activity), a surge that is mediated by the SCN.[43] Thus, circadian rhythms are very stereotypical in rodent mating behaviors; however, humans do not display any equivalent circadian rhythm in mating activity. Despite that phenomenon, there is clinical evidence to suggest that sleep-wake and circadian disruption may play a role in certain human reproductive disorders. For example, shiftwork in pregnant women may be associated with a greater risk of preterm birth,[44] though other analyses dispute this conclusion.[45]

Sleep has also been shown to have an effect on sex hormones, particularly testosterone. Testosterone secretion is linked to sleep cycles, with peak levels occurring in the middle of the sleep cycle, often near the time of REM sleep onset.[46] Insufficient or fragmented sleep, which reduces the amount of REM, blocks the nocturnal increase in testosterone. Thus, sleep disruption could be a risk factor for low testosterone levels.[46]

5. Non-Gonadal Endocrine Factors Impact Sleep

A multitude of different hormones have been shown to impact the sleep state, as well as the quantity and quality of sleep achieved. The key circadian mediator melatonin is one of the best-known and most directly sleep-impacting hormones; it is classically low during the daytime and increases after the onset of darkness, stimulating sleep in humans.[47] Melatonin administration, which is available as an over-the-counter pharmaceutical, has been shown to increase total sleep time and sleep maintenance.[48] Though melatonin may be sedative in some species such as zebrafish,[49] it appears to not have direct sedative effects in humans.[50] Instead, melatonin in humans appears to be a link between environmental cues, most notably light, and the circadian synchronization of biological processes.[51] This

effect is recapitulated pharmacologically, as exogenous melatonin has also been shown to replicate EEG changes from the circadian pacemaker.[52]

Beyond melatonin, the HPA axis has been shown to have an impact on sleep. Administration of corticotropin releasing hormone (CRH), the first hormone in the HPA axis, increases wake time,[53] and conversely, administration of a specific CRH receptor blockade decreases wake time.[54] Similarly, insomnia has been associated with an all-day increase in HPA axis activity and cortisol secretion.[55] However, paradoxically, exogenous glucocorticoids have been shown to be stimulators of slow wave sleep and inhibitors of REM sleep, particularly in the context of the very high cortisol levels of late pregnancy.[56] It has been hypothesized that this effect is due to feedback inhibition by cortisol of CRH release.[57]

6. Biological Sex Differences and Ovarian Steroids Impact Sleep Behavior

As sleep is highly evolutionarily conserved, the suggestion of biological sex differences may be counterintuitive, but reproductive and sex hormones have also been shown to interact with the sleep-wake system. Women and men have long been clinically shown to have differing sleep patterns.[5] In particular, women paradoxically sleep longer than men, but generally self-report a lower sleep quality.[5] Objective data suggest women should have higher sleep quality than men; women have longer total sleep time and less total wake time, a shorter latency to sleep onset, and higher sleep efficiency than men.[58-59] EEG studies have also shown a higher proportion of deep slow-wave sleep (stage 3) and less light sleep (stage 1 and 2) in women than men.[60] However, clinical evidence is not in agreement with those findings, showing that women have been consistently diagnosed with insomnia and other sleep disorders, including RLS, at a markedly higher rate than men.[5,61]

It is unclear if male sex steroids, mainly testosterone, affect sleep in men, as paradoxically both low testosterone levels and testosterone replacement have been shown to be risk factors for sleep deprivation.[46] In animal studies, castration does not significantly change sleep time in male rodents, suggesting little impact of testosterone levels on male sleep.[8] However, there is a larger complement of scientific literature suggesting an impact of female hormones on sleep behavior.

Overall sex differences in sleep patterns may be due to sleep-independent factors, including psychosocial ones, which may be tied to a higher presence of anxiety in females;[5] inversely, sleep loss may also be more potently anxiogenic in females as well.[62] However, there is strong evidence that hormonal complement plays a role; most strikingly, the sex difference in sleep quality emerges in females with puberty[63] and disappears at menopause. There are specific distinctions in the circadian timing of sleep with biological sex and female hormonal state as well. The endogenous circadian rhythm may have an interaction with biological sex, though the mechanism has not been fully explored.[64] However, there is clinical evidence for a sex difference in circadian timing. Premenopausal women go to bed earlier than men and have much earlier melatonin peaks[65] until menopause, when the sex difference in sleep onset disappears but melatonin peaks become even earlier.[5] The mechanisms of these circadian differences are incompletely understood.

There is little evidence to suggest major primary changes in sleep patterns in women between the different phases of the menstrual cycle.[66] However, there are some minor cyclic differences in sleep attributable to differing hormone levels; for example, a specific type of NREM sleep waveform, sleep spindles, are elevated in the post-ovulatory luteal phase of the menstrual cycle.[66] High progesterone and estrogen levels also correlate

with a lower amount of REM sleep.[27] Clinically, the luteal phase has also been shown to be a time of particularly pronounced sleep disruption during menopause.[67] Finally, another sexually-differentiated hormone, prolactin, has also been shown to increase slow wave sleep based on limited studies of patients with hyperprolactinemia.[68] The broader significance of these changes in humans has not fully been established.

Sleep disorders have also been shown to be particularly prevalent in women at times of hormonal flux, including puberty, pregnancy, and menopause. Exogenous hormones, most notably oral contraception, have been shown to affect sleep in young women; oral contraceptive use increases REM sleep and light sleep, while reducing deep slow wave sleep;[69-70] the mechanism for this change is unclear.

Pregnancy has been shown to be a time of high levels of sleep change and disruption, though it is difficult to differentiate direct effects of hormonal change from physiological changes due to growth and development of the fetus. In particular, obstructive sleep apnea has been shown to be likely in high-risk pregnancies[71] and is associated with comorbidities. While there appear to be few changes in melatonin levels or circadian rhythms during pregnancy,[56] changes in levels of estrogen, progesterone, cortisol and oxytocin may contribute to disruption in sleep, particularly the consistent finding of lower REM sleep times in the third trimester.[56] The connection between sleep and pregnancy is bidirectional, as sleep disruption is a risk for adverse pregnancy outcomes, including gestational diabetes and hypertension.[71]

Sleep disturbances are reported by 40–60% of perimenopausal women, with the most common complaint being nighttime awakenings.[66] The exact sleep symptoms present have shown a hormonal correlation; an increase in follicle stimulating hormone was

associated with nighttime awakenings, while a decrease in E2 was associated with difficulty falling and staying asleep.[72] However, there appear to be few objective sleep changes during menopause in polysomnographic studies.[73] Hormone replacement therapies, generally including E2 and progesterone, have shown improvement in self-reporting of subjective sleep quality across multiple studies.[74-75] There is evidence that E2 plays a role in consolidating sleep at night,[5] possibly leading to increased sleep quality with hormone replacement. However, more objective metrics have shown differing effects, with inconsistent findings in objective sleep quality measures with hormonal therapy.[75] These situations may be due to differing hormonal formulations between studies; alternatively, hormone therapy may exert its main impact in relieving the non-sleep symptoms of menopause, particularly vasomotor symptoms,[75] making women more comfortable and sleep easier to obtain.

Apart from the clinical finding that sex steroids may affect sleep behavior and architecture, the mechanisms underlying how sex steroids influence the sleep circuitry remain a significant gap in our knowledge. The use of animal models is critical for advancing our understanding of the potential endocrine–sleep nexus.

7. Animal Studies Show Mechanisms Connecting Female Gonadal Hormones and Sleep

Historically, the majority of sleep studies have been performed in men or male animals,[5] a deeply unfortunate occurrence that has neglected the impact female animal models can have on illuminating ties between ovarian steroids and sleep. The paucity of basic studies investigating sex differences in sleep has resulted in an unclear picture on the nature of those differences. Gonadally intact female rodents generally spend less time in

sleep states compared with males,[76] but females, despite accumulating less total sleep, have more consolidated sleep bouts, consisting of longer bout durations with less state transitions and fewer arousals.[76] Moreover, NREM-SWA, a quantitative measure of sleep intensity, is higher in females during baseline sleep as well as in recovery sleep following deprivation, a finding in agreement with human clinical data.[76] Perhaps more striking is that in the absence of circulating sex steroids, these sex differences in sleep behavior and architecture are eliminated, suggesting that sex differences in sleep are in part dependent on sex steroids.

Sleep patterns in female rats are exquisitely sensitive to the natural fluctuations of ovarian steroids.[76-77] The rodent estrous cycle is analogous to the human menstrual cycle with a period of four days in rats rather than approximately four weeks in humans. The four phases of the estrous cycle are metestrus, diestrus, proestrus, and estrus.[78] On the metestrus day, analogous to menses in humans, levels of E2 and progesterone are low. On diestrus, analogous to the second week of the human menstrual cycle, E2 begins a gradual rise. During proestrus, E2 spikes followed by surges in other hormones, analogous to the similar surges during ovulation in humans. On estrus, the final day of the cycle, E2 levels decrease (but are still higher than metestrus) while progesterone surges. (Fig. 2)

Multiple studies in rats show that during proestrus, when estrogen and progesterone are elevated, sleep time is significantly reduced compared with other phases of the estrous cycle.[79-80] Exogenous hormone replacement is observed to recapitulate this phenotype[8] in both rats and mice. In these studies, estrogen predominately suppresses dark phase sleep and has little or no effect in the light phase. Thus, a key paradigm for studies of hormonal modulation of sleep has been the use of hormone replacement in ovariectomized rodents,[5]

which can provide hormonal stability that bypasses the rapid hormonal changes inherent to the 4-day menstrual cycle in rats.

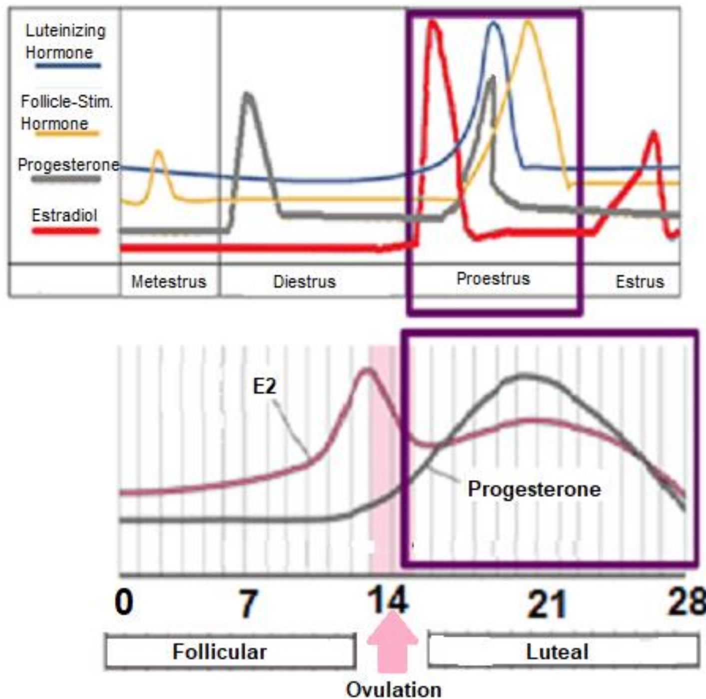


Fig. 2. Comparison of Estrous and Menstrual Cycles. Analogous patterns exist in the timing of E2 and progesterone surges between the four-day estrous cycle in rodents (top) and 4-week menstrual cycle in humans (bottom). Both cycles show an increase in E2 around ovulation followed by an increase in progesterone.

In rodents, estrogens have been broadly shown to increase wake and decrease spontaneous sleep, particularly in the active phase of the light-dark cycle; exogenous replacement of estrogen in females decreases dark phase sleep by 55%.[5] Furthermore, estrogens have been shown to consolidate wake and fragment sleep. However, estrogen-treated rats also have more consolidated slow wave sleep following sleep deprivation by gentle handling,[80] and thus estrogen may be acting to facilitate recovery from sleep deprivation. E2 may interact with the circadian system, as it is shown to have a time-of-day-dependent effect; E2 has been shown to decrease sleep in the active phase and increase it following deprivation in the stereotypical sleep phase.[5,8] Thus, hormonal impact may serve to strengthen circadian entrainment. Supporting that contention, aromatase knockout mice, who are deficient in the formation of estrogen from testosterone, have similar duration of sleep but sleep that is more fragmented and less entrained to the stereotypical (light) phase of the light-dark cycle.

The molecular mechanisms of how hormones impact sleep are poorly understood, and studies investigating where and how female steroids act on the brain are only an emerging area of investigation. Sexual differentiation of the rodent brain occurs during a brief window of early development. Exposure to sex steroids around the day of birth results in the masculinization and defeminization of the rodent brain, while absence of sex steroids leads to a feminization process.[82-83] Adult production of sex steroids cement appropriate behaviors specific to the sex of the animals. Studies in rats have suggested that E2 effects on sleep are established by the first phase of this process, the early programming effects of sex steroids.[8] Female rats exposed to a masculinizing dose of testosterone during the sensitive window for brain sexual differentiation exhibit male-like responsivity to E2 and testosterone in adulthood and exhibited male signatures in the sleep-active Ventral Lateral Preoptic (VLPO) nucleus.[84]

Steroid receptors, particularly for estrogen and progesterone, are present throughout the brain and prevalent on multiple sleep-regulating nuclei such as the hypothalamus[85] and basal forebrain.[86] Previous work in rodents implicates the VLPO in particular as a key site of mediating E2 actions over sleep. In adult ovariectomized females, E2 decreases activation of sleep-active VLPO neurons[7] and downregulates levels of lipocalin-type prostaglandin D synthase (L-PGDS), the enzyme responsible for the production of prostaglandin D2 that potently promotes sleep.[87-88] Estrogens also decrease expression of wake-inhibiting adenosine 2A receptors, suggesting a potential alternate mechanism for an inhibitory impact on homeostatic sleep pressure.[89] However, these findings are complicated by studies showing that the VLPO is not a major site of estrogen sensitivity.[90] Instead, it is an upstream nucleus, the MnPN, which may be most responsible

for mediating E2 action over homeostatic sleep pressure. Blocking E2 action directly in the median preoptic nucleus of female rats attenuates E2 suppression of sleep.[7] Downstream, the wake-promoting system of the lateral hypothalamus receives inputs from the MnPN and VLPO and is highly sensitive to fluctuations in endogenous and exogenous ovarian steroids,[5] suggesting that this section of the homeostatic sleep/wake circuitry may be a key site for estrogen action.

While estrogen receptors are present in the key circadian nucleus of the Suprachiasmatic Nucleus (SCN),[91] there is not a great deal of evidence suggesting an important function for estrogen in circadian rhythms. By contrast, androgens appear to be important to the activity of the SCN, increasing the fealty of certain behaviors to the circadian clock by mediating its response to light.[92-93] Finally, it is important to note certain sex differences may be more impacted by chromosomal complement than hormonal status. Female mice had a higher level of slow wave activity in their active phase than male mice when both were ovariectomized or gonadectomized respectively.[94] Additionally, anatomically female mice engineered to have an XY chromosomal compliment in the "four core genotypes" model acquire more sleep during their active phase and have higher delta power (a measure of sleep efficiency) than XX females, suggesting processes mediating recovery from sleep loss are partially dependent on sex chromosomes.[95] To more fully understand the connections between sleep and endocrine factors, an exploration of the complex neurobiology of sleep behavior itself is necessary.

8. Several Hypotheses Exist as to the Functions of Sleep

Despite its ubiquitous nature, very little is known about why sleep is necessary; from an evolutionary standpoint, the necessity for an animal to spend such a large portion

of its lifespan in a position both vulnerable and seemingly of little use to the animal would seem a poor adaptation. The question of sleep function in animal physiology is a hotly-debated one, with several working hypotheses. In particular, three well-developed hypotheses have formed of key sleep functions: as a method of brain microenvironmental homeostasis, a mechanism for memory consolidation and cognition, and as a regulator of metabolism and energy balance.

The evidence for sleep as a homeostatic process is well-established. Like other homeostatic systems, sleep pressure responds in an analog fashion to the relative distance from its homeostatic mean; in essence, sleep pressure directly increases with wake time. The recently identified glymphatic system may provide a mechanism for control of brain microenvironmental homeostasis that is sleep-dependent. The glymphatic system is a fluid-dynamic model of cerebrospinal (CSF) and interstitial (ISF) fluid flow around the brain and through the brain parenchyma. This flow has been shown to be important for clearance of metabolites and other waste products, including Amyloid Beta[96] from the brain. Additionally, glymphatic flow has been shown to be upregulated by as much as a factor of ten in the sleep state.[96] Thus, a model has emerged in which waste products of metabolism and brain activity build up in the wake state due to inadequate glymphatic flow, but are cleared from the parenchyma in the sleep state when flow is increased.[97] These findings could suggest that brain clearance is a key function of the sleep state and a key purpose of the homeostatic sleep function.

Another hypothesis for the function of sleep involves the process of memory consolidation.[98] Multiple studies have shown that memories are enhanced during sleep; in particular, declarative memories have been shown to be enhanced after NREM sleep,[99-100]

while non-declarative or emotional memories have shown enhancement after REM sleep.[100] Sleep has also been shown to be important for synaptic downscaling, in which synapses are uniformly lessened in strength during sleep.[101] This uniform downscaling prevents or relieves the saturation of synapse receptor patches. Relief of saturation allows for further long-term potentiation and depression at the same synapses, in order for more differentiation of synaptic strength (and thus memory formation) to proceed.

Perhaps most importantly from an endocrine perspective, sleep has been hypothesized as an important mechanism for the regulation of metabolism and systemic energy balance. Overall metabolism declines only modestly in sleep,[102] suggesting that energy conservation *per se* is not a key function of the sleep state. One working hypothesis for the purpose of slower metabolism is that it may allow free radical scavengers more freedom to reduce reactive molecules that can damage the brain.[103] More broadly though, sleep has been shown to induce the fluctuation of hormones important for regulation of normal metabolism. Sleep disruption has been shown to enhance ghrelin[36] and decrease leptin[30] levels, stimulating appetite; it is also associated with a state similar to insulin resistance, possibly due to dysregulation of growth hormone (GH) levels.[104] As a result, disruptions in sleep have been shown to be a risk factor and exacerbating factor for metabolic syndrome and related disorders.[36]

9. Sleep Behavior Consists of Multiple Distinguishable States

Sleep consists of several distinct states, which can be distinguished by their patterns of brain activity.[84] Quantifiable changes in sleep are measured most accurately by monitoring neuronal and muscle activity via electroencephalography (EEG) and electromyogram (EMG), respectively. Each sleep state presents a characteristic EEG/EMG

signature that can be reproducibly back-correlated to determine the sleep state. An automated scoring algorithm has been employed to correlate EEG/EMG signatures and score them as wake, NREM, or REM sleep.

The most important distinction between sleep states is between REM and NREM sleep. NREM sleep predominates at the outset of a particular sleep bout, and is distinguished by an ordering and synchronizing of brain activity.[5] This synchronization leads to a decrease in the frequency and increase in the amplitude of brain waves, causing waves in the delta (0-4 Hz) range to predominate; those waves are considered synonymous with Slow Wave Activity (SWA).[105] During NREM sleep, muscle activity is decreased relative to the wake state, but paralysis of skeletal muscles is not present.[5] In humans, further refinement of NREM sleep can be achieved by separating it into distinct stages, numbered 1-3 in order of increasing depth of sleep. Stage 3 (redefined in 2007 from the prior stages 3 and 4)[106] is referred to as slow wave sleep and represents the deepest sleep states.

In contrast, REM sleep, also known as paradoxical sleep, consists of highly disordered brain activity that somewhat mimics brain activity in the wake state. In this state, waves in the theta (4-8 Hz) range dominate,[107] and skeletal muscles (except the oculomotor muscles) are paralyzed. REM sleep does not occur at the onset of a sleep bout in healthy animals, instead appearing later in the sleep bout after a period of slow wave sleep has been completed.[84] The differing functions of REM and NREM sleep are poorly understood, and to date many sleep studies have focused on the aggregate time spent in sleep versus wake as their main metric. However, methods do exist for isolating REM or NREM sleep. For example, the flowerpot method, in which an experimental animal is

allowed to sleep on a small shelf, such as an upside down flowerpot, above a pool of water, selectively deprives the experimental subject of REM sleep only by prohibiting sleep during periods of muscle paralysis.[108] These methods may become more prominent as further differences between the two states are elucidated.

Beyond defining sleep states, EEG signatures also provide additional information about sleep quality. During NREM sleep, slow wave activity (NREM-SWA, the quantity of EEG waves of frequency 0.5-4Hz normalized to total EEG activity) is a useful signature of the slow wave sleep state.[109] NREM-SWA has been shown to be a measure of the deepest sleep, sleep that can be thought of as efficiently resolving the animal's homeostatic need. Using these EEG/EMG data, we have consistently shown that estrogens alone (endogenous or exogenous E2) are sufficient to reduce the time spent in sleep states with a concomitant increase in the time spent in wake, findings which are in agreement with reports from other laboratories.[6-8,80,110-111] Together these studies suggest that in female rats, E2 mediates normal sleep-wake patterns, providing a model system to study how estrogens modulate the sleep circuitry to elicit changes in sleep behavior.

10. The Circadian and Homeostatic Systems Drive Sleep Pressure

The biological circuitry of sleep is an area of intense inquiry, with many questions remaining on both the neuroanatomy and neurochemistry of the relevant pathways. This question is complicated by the existence of two distinct systems governing aspects of sleep regulation. These systems' net output is generally described as sleep pressure. Sleep pressure has been defined as the intrinsic need for sleep of a given animal at a given time.[112] Beyond the familiar intrinsic feeling of sleepiness as a manifestation of sleep pressure, quantitative markers derived from EEG outputs exist that can approximate sleep pressure

in a reproducible fashion.[5] The two sleep-pressure systems, known as the circadian wake system and the homeostatic sleep pressure system, operate in parallel and in concert to generate an overall sleep pressure that is responsive to both the animal's intrinsic homeostatic needs as well as external factors such as the light-dark cycle.

The better-understood of the two systems which combine to govern sleep pressure is the circadian wake system. The circadian system orients sleep to the light and dark cycle, as well as consolidates sleep and wake into larger blocks. Circadian timing has two key properties. First, it has an endogenous rhythm with a period of approximately (though not exactly) 24 hours.[113] Second, that rhythm can be shifted in response to external cues.[113] Light-dark cycles are both the most prevalent and potent of these cues, but other factors such as exercise, feeding, temperature, and certain pharmacological agents have been shown to entrain the system as well, in some cases maladaptively.[113] The key neurobiological regulator of the circadian sleep system is the suprachiasmatic nucleus (SCN) of the hypothalamus. Animals with lesions of the SCN have been shown to have as much total sleep time as controls, but sleep in unconsolidated random bouts unrelated to the light-dark cycle.[114] Transplantation of a donor SCN has been shown to rescue a normal phenotype in that regard.[115] Additionally, studies of animals isolated from the natural light-dark cycle and left to sleep *ad libitum* show that those humans settle into a diurnal sleep pattern that approximates, but does not exactly mimic, the 24-hour day, showing the intrinsic rhythmicity of the SCN.[116] The SCN receives its principal entraining inputs from environmental light cues through specialized photosensitive ganglion cells in the retina.[117] Importantly, the photoreceptors and cortical areas responsible for conscious vision are not involved, meaning that the circadian rhythm is reasonably well entrained in most blind

animals.[118] Importantly for the endocrine system, the circadian system serves to create "biological day" and "biological night". Hormones and other biological properties have been shown to fluctuate on a 24-hour cycle according to stereotypical patterns; lesions of the circadian system have been shown to disrupt the daily fluctuation of hormone levels such as cortisol,[30] growth hormone,[41] and leptin,[30] among others. (See section I-4.)

The circadian system is highly centralized, with the SCN being the key center of its action. The SCN exerts circadian control in myriad ways, both neurologically and hormonally. Neurons in the SCN are home to a set of clock transcription factors that stimulate their own repressors,[119] forming a daily oscillating cycle of gene expression. By a mechanism not fully understood, this mechanism governs the differing release of neurotransmitters depending on the time of the cycle.[120] Thus, the SCN releases different waves of neurotransmitters, such as glutamate, GABA, and vasopressin.[121] The main neurological projections of the SCN radiate to the medial hypothalamus, but for hormonal control, the epithalamus is a key site of SCN effects. The pineal gland of the epithalamus releases melatonin, perhaps the best-known hormonal circadian modulator. Release of melatonin is dependent on environmental inputs, most notably the light cycle; melatonin increases several hours after the onset of the dark phase of the light cycle, and remains high until the restoration of the light phase the next day.[47] While melatonin is not required for the initiation or maintenance of circadian rhythms overall,[51] it serves as a key link to entrain biological circadian processes to light cues,[122] and may stimulate sleep in humans and some animal species, possibly through adenosine signaling.[49, 51] In humans, melatonin effects are the principal mediator for normal sleep onset several hours into the dark phase of the diurnal cycle. Melatonin receptors have been shown to exist in myriad tissues,[123]

potentially providing a system-wide mechanism for circadian synchronicity that drives an acute need for sleep at a stereotypical time each day.

The second sleep system, quite distinct from the circadian system, is the homeostatic pressure system. As the name suggests, the homeostatic sleep system governs the amount of sleep needed after a given period of wake to maintain homeostasis.[124] The total amount of sleep needed for an animal in a given period of time tends to be quite consistent, and independent of both the circadian system and the light-dark cycle.[124] This phenomenon is further exemplified by the need for recovery sleep, which is nearly always necessary after periods of sleep deprivation.[125] Similarly to other homeostatic systems such as temperature, extreme loss of homeostasis (such as through prolonged sleep deprivation) is fatal.[126] Homeostatic sleep pressure increases roughly linearly with increasing wake time, reaching a maximum at the onset of the sleep state, and then decreases roughly linearly with time spent asleep.[124] There is debate over whether homeostatic sleep pressure has a measurable biological correlate, though studies have shown correlations with both molecular markers such as adenosine[127-128] and behavioral markers such as delta power during recovery NREM sleep.[129]

The two pathways, circadian and homeostatic, work in concert to generate an overall sleep pressure. Homeostatic sleep pressure increases monotonically throughout the day; however, during the daylight hours in humans, circadian wake drive is also high and increasing. By the onset of the dark phase of the cycle, both homeostatic sleep drive and circadian wake drive are high, cancelling the effect of either. However, after the onset of the dark phase, circadian wake drive begins to decrease, while homeostatic sleep drive remains high, stimulating the onset of the sleep state. By the end of the sleep state in the

early morning hours, circadian wake drive is still low, but homeostatic sleep drive is low enough to compensate, causing the switch to flip again and the onset of the wake state to commence. (Fig. 3) This dual cycle has important impacts on situations where sleep occurs outside of the normal rhythm; for example, in shift workers, falling asleep in the early morning is not a problem as circadian wake drive is low and sleep pressure is high.[130] However, remaining asleep through the day can be difficult as circadian wake drive increases while sleep pressure falls.

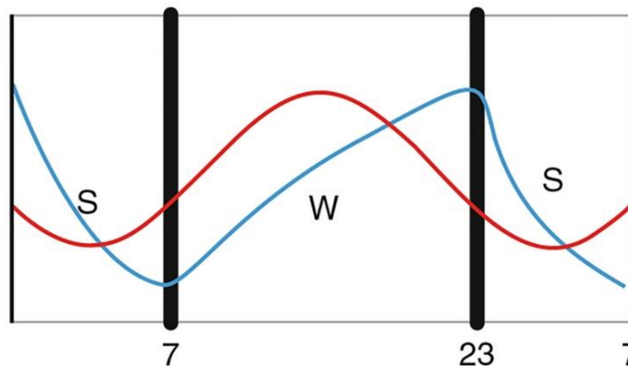


Fig. 3. Schematic of the Circadian and Homeostatic Systems. The homeostatic sleep process (blue) creates sleep pressure roughly linearly in response to time awake, and decreases with time asleep. The circadian wake process (Red) is most active during the daylight hours and provides a method of maintaining wake. After sunset, melatonin stimulates a breakdown in the circadian process, which allows the pro-sleep homeostatic process to become dominant, stimulating the sleep state. After a period of sleep, homeostatic pressure has lowered enough that even a low circadian wake drive becomes dominant, stimulating the wake state.

11. Diverse Chemical Mechanisms Govern Homeostatic Sleep Pressure Systems

Unlike the circadian system, which is largely dependent on environmental inputs via the specialized retinal ganglion cells, the homeostatic system appears to have multiple inputs. An entire class of molecules known as somnogens have been identified that appear to increase homeostatic sleep pressure. Among the most important of these molecules is the nucleoside adenosine,[131-132] produced in the brain both purposefully as a neurotransmitter and as a waste product of ATP metabolism. Several other molecules, such as prostaglandin D2,[133] IL-1,[134] and TNF-alpha,[135] have been hypothesized to act in similar fashions to stimulate the sleep pressure homeostat. Additionally, an emerging area of

research in mechanisms of the homeostatic sleep pressure system may be through the process of protein phosphorylation. A family of proteins, Sleep Need Index Phosphorylation Proteins (SNIPPs), have been found to become steadily phosphorylated during wake and dephosphorylated during sleep.[136] The kinase Sik3 has been shown to aid in this phosphorylation; a constitutively active mutant of this kinase has been shown to induce sleep pressure artificially, resulting in mice with higher sleep times and delta power during their NREM sleep.[137] Downstream of these somnogen initiators, the homeostatic sleep pressure system contains multiple neurotransmitters, including orexin, acetylcholine, monoamines, and glutamate.[138]

Sex hormones have been shown to interact with sleep pressure systems. Interactions have been shown between E2 and the circadian system, including shortening the period of circadian rhythms;[139] however, connections between gonadal hormones and circadian timing appear to be stronger in rodents than humans.[5] Interactions have also been shown between sex hormones and the homeostatic sleep pressure system, as levels of the somnogen adenosine were seen to increase in females during proestrus.[111]

12. Adenosine is an Important Marker and Mediator of Sleep Pressure

Sleep pressure has been described as the intrinsic need for sleep of a given animal at a given time.[112] Homeostatic sleep pressure is the concept that an organism requires a set amount of sleep in a given time period, and the brain has an intrinsic measure of sleep imbalance and need.[140] Sleep homeostasis is carefully regulated; like homeostatic imbalances in body temperature or pH, extreme lack of sleep can be fatal.[126] Neurochemically, a wide variety of molecules have been identified as affecting homeostatic sleep pressure. However, one of the most ubiquitous somnogens is the

nucleoside adenosine. Indeed, many laypersons are familiar with the hypnogenic effects of adenosine through the widespread use of the non-specific adenosine receptor antagonist caffeine.[141] Adenosine is a ubiquitous biological substance; it is produced both ubiquitously as a byproduct of adenosine triphosphate (ATP) metabolism and purposefully in response to stimuli. It is produced both intracellularly and extracellularly. Intracellularly, adenosine is produced by the breakdown of adenosine monophosphate (AMP) by cytosolic 5′-nucleotidase.[142] Extracellularly, adenosine is produced through ectonucleotidase mediated hydrolysis of released ATP and AMP. The final step is AMP hydrolysis by 5′-ectonucleotidase to adenosine.[143] Adenosine breakdown may occur through several pathways. It may be metabolized to AMP by adenosine kinase,[144] to inosine by adenosine deaminase (ADA),[145] or to *S*-adenosyl homocysteine (SAH) by hydrolases.[146]

Multiple cell types in the brain have been shown to be involved in adenosine production and degradation. Neurons have been shown to produce adenosine in response to glutamate stimulation[147] and oxygen or glucose deprivation,[147] while microglia have been shown to produce adenosine in response to oxidative stress.[147] Astrocytes are also important sites of adenosine production. Limited studies have shown an interaction between adenosine production and E2; in the female hippocampus, E2 has been shown to upregulate CD73, the exonuclease which breaks down AMP to adenosine.[148]

Though adenosine signaling has been shown to have multiple functions, somnogenic effects are a key property of the system. Adenosine has been shown to accumulate in the brain[149] with increasing wake time and decrease in the sleep state.[149] During wake, breakdown and clearance of adenosine, particularly by enzymes such as adenosine deaminase, is insufficient to keep up with production, and levels rise.

Conversely, during sleep, production decreases relative to degradation and clearance, and levels decrease. Adenosine has been hypothesized as a dual marker and mediator of sleep pressure; however, there likely exist multiple sites of action. It has long been shown that adenosine accumulates with wake time in the basal forebrain,[149] and adenosine reduces firing of basal forebrain neurons *in vitro*.[150] However, lesion studies of the basal forebrain have shown that adenosine action in that area is not sufficient to drive sleep behavior.[149] Conversely, there may be evidence that adenosine levels activate as a mediator of homeostatic sleep need in the preoptic area. Low (nanomolar) concentrations of adenosine have been shown to enhance wake neurotransmission due to activation of the inhibitory A1 receptor in the preoptic hypothalamus, while high (micromolar) adenosine concentrations have been shown to inhibit wake neurotransmission through activation of stimulatory A2A receptors in the same nuclei.[151-152]

Four adenosine receptors have been identified, A1R, A2AR, A2BR, and A3R.[153] However, only two of the four, the inhibitory Gi-coupled A1R and the stimulatory Gs-coupled A2AR, have been found to be widely expressed in the nervous system.[154] It is widely accepted that adenosine sleep-inducing actions are mediated through the A1R and A2AR present in key sleep and arousal nuclei.[155] In particular, the Ventral Lateral Preoptic (VLPO) and Median Preoptic (MnPN) Nuclei have been shown as sites of sensitivity to adenosine as a mediator of homeostatic sleep pressure.[151] E2 has been shown to downregulate expression of A2A receptors in the VLPO,[89] which activate sleep-promoting cells and networks. The role of the A1 receptor in sleep appears to differ by brain location. Activation of A1R has been shown to stimulate sleep in brain areas like the basal forebrain, and it has also been hypothesized to modulate sleep activation more than induce it.[156] In

the sleep-active preoptic hypothalamus, evidence appears to point to A1R and A2AR being counteracting components, with differences in adenosine content in the hypothalamus playing a role in sleep pressure by activation of A1 and A2A receptors. However, behavioral and physiologic effects of interactions between E2 and adenosine have not yet been explored. Thus, we propose the premise that interactions between E2 and adenosine provide a mechanism for E2 effects on homeostatic sleep pressure.

13. Homeostatic Sleep Circuitry is Governed by a Number of Neuroanatomical Loci

The sleep circuitry is a complex and multi-faceted system from a neuroanatomical perspective, with separate wake-promoting and sleep-promoting networks. Studies of wake-promoting systems historically focused on monoaminergic and peptidergic neurons of the upper brainstem. These wake-promoting systems exhibit high levels of diversity in their site of action and neurotransmitter usage. There have been no less than five distinct monoaminergic and peptidergic wake systems identified: Noradrenaline projections emanate from the locus coeruleus, 5HT from the raphe, acetylcholine from the pedunculopontine and laterodorsal tegmentum, histamine from the tuberomammillary nucleus, and orexin from the lateral hypothalamus.[157] These systems project in an anatomically ascending manner, broadly reaching the cortex by way of the thalamus, ventral portions of hypothalamus, and basal forebrain. Paradoxically though, lesions of these pathways had little effect on total sleep and wake time.[158] Thus, in recent years, the importance of glutamatergic and GABAergic networks on the wake-promoting circuitry has grown. GABAergic inputs from the basal forebrain, lateral hypothalamus, and supramammillary nucleus have been shown to promote wake, as have glutamatergic inputs from the supramammillary, parabrachial, and pedunculopintine nuclei.[157] (Fig. 4)

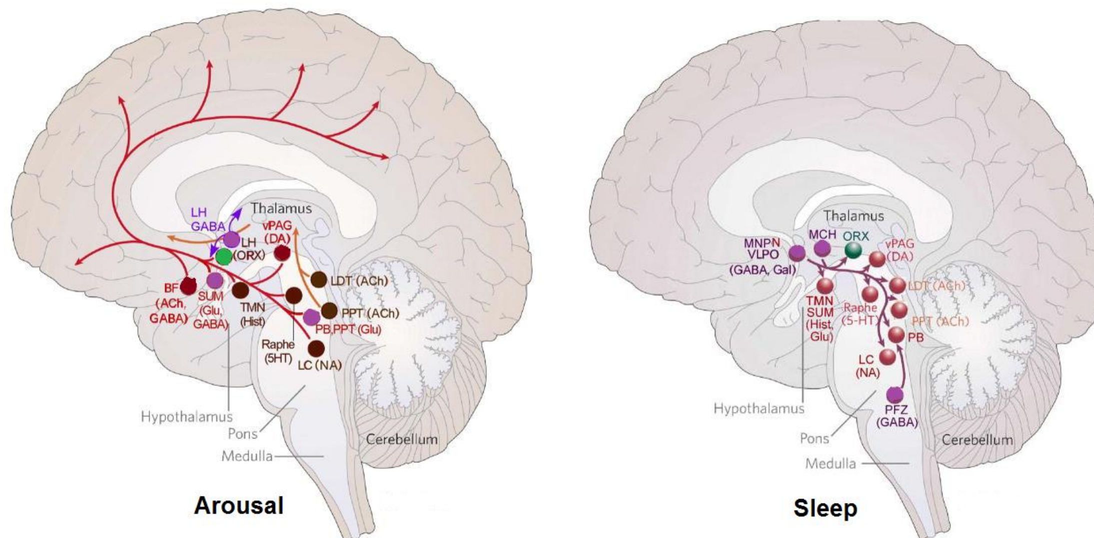


Fig. 4. Functional Anatomy of Sleep and Wake Pathways. Modified from Saper CB and Fuller PM 2017.[157] A diagram summarizing the neurotransmitter systems that appear to play major roles in promoting wake and sleep. Glutamatergic/GABAergic systems are in purple, orexinergic in green, and other neurotransmitters in red/burgundy. The backbone of the arousal system is the glutamatergic input from the parabrachial nucleus (PB) and pedunculopontine tegmental nucleus (PPT) to the basal forebrain, and the GABAergic and cholinergic neurons in the basal forebrain (BF) that diffusely innervate the cerebral cortex. In the sleep state, Ventrolateral preoptic (VLPO) and median preoptic (MnPN) GABAergic neurons send axons to most components of the arousal system, and are thought to inhibit them in a coordinated fashion. Parafacial zone (PFZ) GABAergic neurons in the medulla induce sleep mainly by inhibiting the parabrachial glutamatergic arousal neurons. Melanin-concentrating hormone (MCH) neurons in the lateral hypothalamus contain both GABA and glutamate, and may be able to release them at different terminal sites. Abbreviations: 5HT, serotonin; Ach, acetylcholine; DA, dopamine; Hist, histamine; LC, locus coeruleus; LH. lateral hypothalamus; LDT, laterodorsal tegmental nucleus NA, noradrenaline; ORX, orexin; SUM, supramammilary; TMN, tuberomammillary nucleus; vPAG, ventral periaqueductal gray.

14. The Preoptic Area is an Important Locus of Sleep Networks

Several nuclei are thought to stimulate sleep in the homeostatic sleep-pressure system, with two nuclei of the preoptic hypothalamus of key importance. The VLPO and MnPN are thought to be key originators of this pathway [5, 159-160] A feed-forward loop has been identified in which the MnPN both inhibits the wake system of the lateral hypothalamus and stimulates the VLPO, which itself serves as an inhibitor of the wake

nuclei in the lateral hypothalamus.[160] More broadly, these nuclei send descending GABAergic projections to the brainstem that inhibit many of the same wake-driving nuclei described in the ascending wake system.[157] Particular targets include the supramammilary, tuberomammilary, and parabrachial nuclei, as well as monoaminergic nuclei such as the raphe and locus coeruleus. Additionally, projections from the MnPN and VLPO stimulate sleep by way of other brainstem nuclei such as the ventral periaqueductal gray.[157] The MnPN and VLPO have been shown to receive circadian inputs from the SCN by indirect projections via the dorsal medial hypothalamus and/or supraventricular zone, suggesting a potential pathway for the integration of the circadian and homeostatic systems.[161-162]

A particular target of the MnPN and VLPO descending sleep circuit is the orexinergic wake system of the lateral hyptothalamus. Orexinergic wake nuclei have widespread projections to the cortex and brainstem and are primarily active during the wake phase;[163] lesions of these nuclei have been shown to induce a narcoleptic-like phenotype.[164] The orexin system is important from an endocrine perspective in its dual importance to both sleep-wake circuitry and feeding behavior. Orexin projections from the lateral hypothalamus project broadly across the brain to centers important for feeding, such as the paraventricular nucleus, and centers important for maintenance of wake, such as the locus coeruleus.[165] However, the exact structure and function relationship of these pathways, and what neuronal pathways may exist connecting between feeding and sleep behavior, is incompletely known.

The VLPO and MnPN send GABAergic projections to key mediators of the wake state, including nuclei in the lateral hypothalamus governing the orexinergic wake system.[5] In particular, The MnPN contains GABAergic sleep-active projection neurons, which

innervate the lateral hypothalamus and multiple brainstem nuclei.[166] GABAergic MnPN and VLPO neurons have direct inhibitory control over the orexinergic neurons in the perifornical area/ lateral hypothalamus.[167] These orexinergic neurons are a key source of arousal signaling, suggesting a sleep-promoting mechanism of the MnPN, which also innervates the VLPO[168] in a feed-forward loop. (Fig. 5) Numerous studies have shown neurons in the VLPO and the MnPN are involved in sleep-regulatory mechanisms. [167,169-171] Both the VLPO and MnPN (i) have a predominant number of sleep-active cells (i.e., the number of Fos-ir neurons increase following episodes of sleep but not waking) [84,160] (ii) have a high concentration of neurons with elevated discharge during sleep compared to waking (i.e. sleep-active discharge pattern),[172] and (iii) are thought to promote and sustain sleep by inhibiting arousal centers.[166,169-171] As the MnPN and VLPO serve as upstream modulators of the orexinergic wake system, direct manipulations of these nuclei can therefore be employed to test the subsequent sleep-wake pathway.

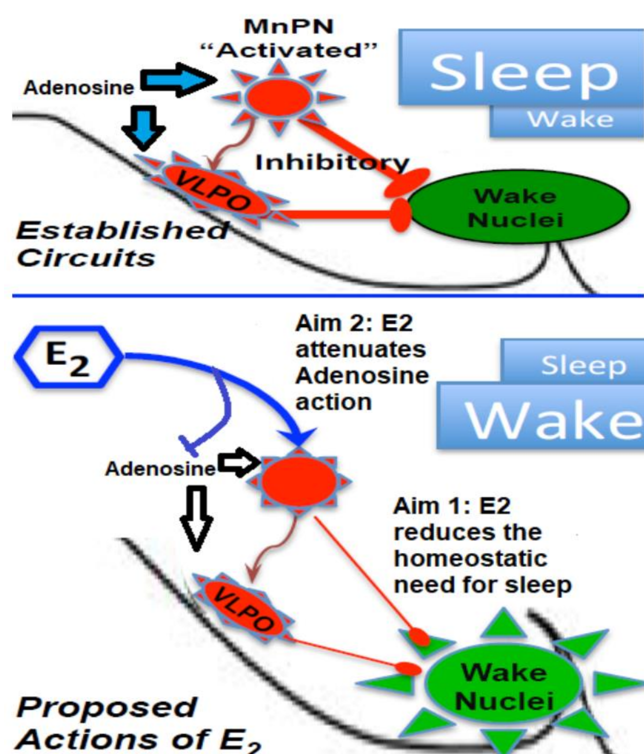


Fig. 5. E2 and Adenosine Act on MnPN-VLPO Circuitry. Adenosine stimulates sleep-active centers of the MnPN and VLPO to activate their projections to downstream wake nuclei. With E2 present, interactions with adenosine reduce MnPN activation, relieving inhibition on wake nuclei and stimulating the wake state.

Using Fos immunoreactivity (Fos-ir) as a marker for neuronal activation, we found that E2 reduces the number of Fos-ir cells in the VLPO and MnPN in adult

ovariectomized (OVX) females during the sleep phase. Since activation of sleep-active neurons in the VLPO and MnPN has been shown to be proportional to accumulated sleep, our findings suggest that E2 action in the VLPO and MnPN suppresses activation of sleep-active cells, prolonging wake.

15. Sleep Disorders Have a Broad Clinical Impact

Sleep disorders are both quite prevalent and generally thought to be underdiagnosed. While we will not attempt to give an exhaustive overview of all sleep disorders here, this section will profile some common conditions to illustrate the myriad interactions with the endocrine system. Sleep disorders can take two forms: disorders of sleep quantity and quality *per se*, and related comorbidities which can be introduced or exacerbated by sleep disruption.

Insomnia is the most common sleep disorder; it is defined as the persistent inability to sleep despite the opportunity to do so. It has been estimated that insomnia is clinically present in 6% of the population, and as many as a third of individuals may show some symptoms.[4] Insomnia may be a primary condition, or secondary to a multitude of other neurological, psychiatric, and physical disorders. A multitude of pharmaceuticals and non-pharmaceutical interventions exist to combat insomnia, of varying effectiveness. Importantly for the endocrine field, insomnia shows a sex difference, as it is significantly more common in women than men.[5] Additionally, insomnia pharmacology is an arena where sex differences have become a prominent issue. One of the most common anti-insomnia medications, the benzodiazepine-like drug zolpidem (popularly known by the brand name Ambien), was also the first major drug to show a strong interaction with the patient's biological sex, as in 2013 the FDA reduced the recommended dose for women to

half that of men. Though the case of zolpidem sexual interaction was due to differing rates of liver metabolism,[173] the brain has shown an interaction between pharmacology and biological sex in the context of insomnia as well. A study administering olanzapine (a second generation anti-psychotic) showed sex differences in its effect on sleep, as slow wave sleep increased in women and decreased in men.[174]

Another prominent sleep disorder is restless legs syndrome (RLS). RLS is the uncontrollable urge to move one's legs when at rest, which leads to an inhibition of deep sleep states.[175] RLS is quite common, with estimates of between 2% and 15% of the population displaying symptoms. The causes are unclear, but several underlying diseases and medication side-effects, most notably iron deficiency,[176] have been speculated as potential causes. Similar to insomnia, RLS is far more prevalent in women than men, for reasons that remain unclear.[61]

A prominent sleep disorder about which more is known surrounding the etiology is obstructive sleep apnea (OSA). OSA is marked by closure of the airway during the night, leading to periods of cessation or attenuation of breathing and hypoxia.[177] OSA is often under-diagnosed, as it presents with very non-specific symptoms, such as daytime sleepiness, fatigue, and impaired cognition, to the sufferer. As such, it is often only noticed by bed partners due to nighttime snoring.[178] OSA is often a comorbidity of obesity, due to the presence of additional fatty tissue in structures surrounding the airway.[179] Unlike many sleep disorders, OSA is more commonly diagnosed in males,[178] though there is speculation that it may simply be underdiagnosed in women. OSA is usually managed mechanically through the use of continuous positive airway pressure (CPAP) machines or mandibular splint devices, which both physically open the airway.[180]

Disorders of circadian regulation are also widespread. Delayed sleep phase disorder (DSPD) is a chronic circadian dysregulation that pushes the onset of sleep and the onset of wake much later relative to societal norms,[181] often due to genetics.[182] It is a form of “social jet lag”, a broader term that also encompasses a delayed circadian phase due to behavioral and environmental factors.[183] DSPD and social jet lag are relatively rare in adults, with a prevalence of under 2 in 1000, but are common in adolescents, with studies showing a prevalence of 5%184 and some estimates being even higher. Treatments of DSPD with melatonin[48] and analogues have shown some success in combating the symptoms, though relapse can be a concern.[185] The converse of this condition, advanced sleep phase disorder (ASPD), is significantly rarer, though more common in the elderly. Both DSPD and ASPD have shown a strong genetic component in familial studies.[182]

Mood disruptions have shown a sleep and circadian component. Disruption of the circadian rhythms of melatonin has been linked to seasonal affective disorder (SAD), a common form of depression. SAD is prevalent in the winter months, when daylight cycles are shorter and cause disruption of melatonin secretion;[186] some SAD patients experience melatonin secretion well into the morning hours, when levels should be low. Morning bright-light therapy to resynchronize melatonin levels has been shown to mitigate some effects of SAD.[186] Major depression has also been linked to melatonin release, and some melatonin receptor agonists have been approved to treat depression.[187]

Sleep disruption has been shown to be tied to many serious pathologies, both cognitive and physical. Sleep changes have been shown to be correlated with Alzheimer’s pathology as well as a potential leading sign of the disease.[188] Alzheimer’s patients have shown decreased sleep at night and increased sleep during the day, as well as an overall

loss of REM sleep.[189] Additionally, self-reported sleep problems, most notably sleep fragmentation, have been associated with a higher risk of Alzheimer's years later.[190] The loss of sleep-dependent microenvironmental homeostasis may be a contributing factor to the accumulation of brain metabolites in such dementia. Amyloid Beta, the key protein which aggregates in Alzheimer's, has been shown to display a diurnal rhythm that increases during wake time and decreases during sleep.[191] The impact of sleep on memory formation and consolidation may also explain portions of this connection.

The strong endocrine connections between sleep and metabolism also present a possible explanation for population-level correlations between sleep disruption and the key public health issue of metabolic syndrome. Obesity is a key public health concern, and sleep has been shown by multiple studies to both impact and have an impact on metabolic and feeding behaviors. Most notably, obesity is a major risk factor for OSA[179] as described above. Conversely, sleep loss has been shown to have a stimulating effect on appetite[192] and has been correlated with increased obesity. However, the molecular mechanisms of these interactions, particularly the connections between clinical phenotypes and neuroendocrine mechanisms, are still ill defined and multi-faceted. Sleep disruption has been shown to increase oxidative stress,[103] enhance pro-inflammatory mediators such as IL-1 and TNF-alpha,[134-135] activate the sympathetic nervous system,[193] and stimulate cortisol secretion.[35] Activation of these pathways have been shown to be risk factors for obesity, metabolic syndrome, and sequelae such as type 2 diabetes.

16. Conclusion and Hypothesis

Sleep behavior demonstrates myriad neuroendocrine interactions and has broad implications for human health. While there is much that is unknown about the reasons for

sleep, evidence exists that it is important for homeostasis of a diverse array of biological functions. Disorders of sleep regulation are extremely prevalent and both a major cause of primary morbidity and an exacerbating factor to many health conditions. Sleep and the endocrine system exhibit a bi-directional interaction, with sleep behavior having a strong influence on endocrine factors and endocrine factors reciprocally influencing sleep behavior. In particular, biological sex and sex hormones have been shown to have a significant impact on sleep function. Unfortunately, until recent years much research on sleep behavior largely disregarded its endocrine connections. Use of both clinical studies and rodent models to investigate interactions between neuroendocrine function, including biological sex, and sleep therefore presents a promising area of further exploration.

In particular, the highly reproducible effects of E2 on sleep in female rodents provide an informative bioassay to investigate how E2 may be affecting sleep mechanisms. Using adult female Sprague-Dawley rats, our studies consistently demonstrate that sleep time is significantly reduced when endogenous ovarian steroids or exogenous E2 are elevated in females but not males. Our data suggest that E2 action at the MnPN, inhibiting sleep-active neurons, is both necessary and sufficient for this effect. Moreover, we have demonstrated that E2 is capable of marked suppression of sleep under sleep deprivation, when sleep pressure is increased. These observations suggest that E2 may alter the homeostatic need for sleep. Our data further suggest that E2 increases the levels of adenosine in the preoptic area, while attenuating the action of adenosine signaling at the sleep-promoting A2A receptor, resulting in reduced sleep duration. These data suggest that there is an interplay between E2 and adenosine at the level of the MnPN which modulates the ability of adenosine to generate sleep pressure. (Fig. 6)

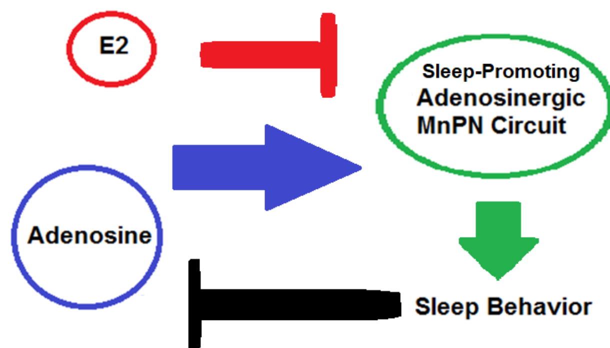


Fig. 6. Schematic of Overall Hypothesis. The overall purpose of this thesis is to the hypothesis that estrogenic modulation of sleep pressure requires the attenuation of adenosine signaling efficacy at the Median Preoptic Nucleus.

This thesis tests the hypothesis that estrogenic modulation of sleep pressure requires the attenuation of adenosine signaling efficacy at the Median Preoptic Nucleus. Understanding interaction of E2 and adenosine may illuminate ties between hormone levels and sleep, and provide significant opportunities for sleep disorder treatments that take female sex and hormonal state into account.

B. General Methods

1. Animals.

Adult female Sprague–Dawley rats (250-350g) were purchased from Charles River Laboratories (Kingston, N.Y.) and housed in the Laboratory Animal Facilities at the University of Maryland, School of Medicine under a reversed 12 h: 12 h dark: light cycle (Lights on at 9PM for Chapter II and experiments 1 and 3 in Chapter III, lights on at 6AM for Chapter III, experiment 2.) with food and water available ad libitum. In all experiments, zeitgeber time 0 (ZT 0) represents Lights ON. Due to logistical limitations, all experimental procedures were run in multiple cohorts, with all experimental groups represented in each cohort. All procedures were performed in accordance with the National Institutes of Health guide for care and use of laboratory animals. All experiments were approved by and were in accordance with the guidelines of the University of Maryland Institutional Animal Care and Use Committee.

2. Gonadectomies and Transmitter/Cannula Implantation.

All surgeries were conducted under isoflurane anesthesia. All animals were ovariectomized (OVX) or castrated according to standard protocol and simultaneously implanted with TL11M2-F40-EET transmitters (Data Sciences International, St. Paul, Minn.). Briefly, animals were OVX using a dorsal incision followed by isolation and removal of the ovaries bilaterally. Males were castrated using a similar dorsal incision followed by isolation and removal of the testes. Using the OVX incision for females or with a separate dorsal incision for males, a bipotential- lead transmitter (DSI Inc., St. Paul, Minn.) was implanted intraperitoneally. Another incision was made along the midline of the head and neck to expose the skull and neck muscle. Two burr holes were drilled asymmetrically and stainless-steel screws (Plastics One, Roanoke, Va.) were implanted at 2 mm anterior/1.5 mm lateral and 7 mm posterior/1.5 mm lateral relative to the bregma. The four transmitter leads were threaded subcutaneously to the head incision. Electroencephalographic (EEG) leads were wrapped around the screws and secured with dental cement. Electromyogram (EMG) leads were implanted directly in the dorsal cervical neck muscle, approximately 1.0 mm apart, and sutured in place.

For experiments that required local injections into preoptic area nuclei (Chapter II experiments 2 and 3, Chapter III experiments 2 and 3) guide cannula were implanted. Three types of guide cannula were used. For MnPN infusion, a single guide cannula (C315G, 26-gauge; Plastics One) targeted to the MnPN was implanted at a 9° angle at the stereotaxic coordinates 0.45mm posterior/ +1.0mm lateral/ 6.5mm ventral relative to bregma. For microdialysis (Chapter III experiment 2), the same posterior and lateral MnPN coordinates were used, with a depth of 6mm using a microdialysis cannula (SciPro Inc. #MAB-6.14.G,

Sanborn, N.Y.). For VLPO infusion (Chapter II experiment 2), a bilateral guide cannula (C235G, 26-gauge; Plastics One) targeted to the VLPO was implanted at the stereotaxic coordinates 0.1mm posterior/ 1.0mm lateral/ 7.0mm ventral relative to bregma. In all cases, the cannula and EEG leads were secured together with dental cement. Upon insertion of the cannula, the opening was closed with a matching dummy provided by the respective cannula manufacturer. The skin along the head was sutured around the guide and dummy cap, and the dorsal incision was closed with wound clips. Fig. 17 and 42 are representative images of the guide cannula placement. All animals were treated with antibiotic ointment and topical lidocaine as well as carprofen (5 mg/kg) postoperatively and then allowed 7 days to recover before the start of the experiments.

3. Data Collection and Sleep Scoring.

Home cages with the telemeter-implanted animals were placed on receiver bases that continuously collected EEG and EMG data at 500Hz and transferred the data to a PC running Dataquest (Chapter II experiments 1-2) or Ponemah (Chapter II experiment 3 and Chapter III) software (both DSI Inc., St. Paul, Minn.). Digitized signal data was scored off line using NeuroScore DSI v3.3.9 (DSI Inc., St. Paul, Minn.). The EEG/EMG signals were parsed into 10 second epochs. A Fast Fourier transform (Hamming window, 4096 samples) was used to break down the EEG frequency bands (Delta (0.5-4Hz), Theta (4-8Hz), Alpha (8-12Hz), Sigma (12-16Hz), Beta (16-24Hz) Gamma (24-50Hz) and Total (0.5-50Hz)). The mean of the absolute value was calculated for the EMG data (bandpass 20-200Hz). These data were exported to Matlab (Matlab R2015, Mathworks, Natick, Mass.) where vigilant states were automatically scored using a custom program developed by the lab (DJP). In the automated program, scoring decisions were based on threshold levels of EEG

delta power, theta power, the ratio of delta to theta, and the EMG activity. Data were normalized to the mean of the entire recording, then the median for each signal was used as a threshold (see table 1 below). The scoring data was reimported into NeuroScore using a custom Matlab program courtesy of Dr. Michael Rempe. The automated scores were visually confirmed in NeuroScore and changes were made where necessary. Table 1 summarizes the gestalt paradigm used to assign sleep states, with determinations of low and high made to each animal's median value for the given parameter. The NeuroScore program was used to compile total time spent in wake, NREM, and REM sleep.

The scored epochs were summed over the 12h dark phase and reported as the total time (in minutes) spent in each state (wake, total sleep, NREM sleep, and REM sleep). The percent change induced by E2 was calculated for each vigilance state: Percent change from oil baseline = [(estradiol_time – baseline_time) / baseline_time] x 100.

EMG	**Delta (0.5-4Hz)**	**Theta (4-8Hz)**	**Sleep State**
High	High **OR** Low	High **OR** Low	**Wake**
Low	High	High	**NREM Sleep**
Low	High	Low	**NREM Sleep**
Low	Low	High	**REM Sleep**
Low	Low	Low	**Wake**

Table 1. Scoring Paradigm. This table describes sleep state scored by EEG/EMG inputs. Determinations of low and high are made relative to each animal's median value for the given parameter.

4. EEG Spectral Analysis.

NREM Slow Wave Activity (NREM-SWA; a marker of sleep homeostasis), was assessed via EEG spectral distributions of NREM sleep bouts. Here, the scored bouts of NREM sleep were imported into Matlab. EEG power spectra were computed between 0.5 - 20Hz in 0.25Hz stepwise bins. Each power bin was normalized to the mean total power from the 24-hour baseline recording, then averaged into 6-hour epochs (ZT times 0-6, 6-12, 12-18, and 18-0).

II. Estradiol Action at the Median Preoptic Nucleus is Necessary and Sufficient for Sleep Suppression in Female Rats

A. Introduction

We have reported in adult female rats that sleep-wake behavior and neuronal activation in preoptic area sleep nuclei are highly sensitive to fluctuations in circulating E2. Sleep behavior in males, is completely insensitive to changes in gonadal steroids, both E2 and testosterone, due to developmental programming effects of gonadal steroids on the preoptic area sleep active nuclei.[6,196] Thus, the preoptic area suggests itself as a potential site of E2 action on sleep. The ventrolateral preoptic area (VLPO) and the median preoptic nucleus (MnPN) are two key sleep-active nuclei involved in the onset and maintenance of sleep.[159-160] Moreover, previous findings suggest sleep-active neurons in the VLPO are sensitive to fluctuations in ovarian steroids.[7] E2 replacement following ovariectomy reduces neuronal activation of VLPO sleep active neurons as well as mRNA expression and protein levels of lipocalin-type prostaglandin D synthase (L-PGDS), the synthesizing enzyme for the somnogen prostaglandin D2.[7,197] Together, these data suggest that E2 action in the preoptic area nuclei may alter critical factors involved in sleep and sleep homeostasis.

In the current study, we sought to expand our knowledge of the mechanisms through which ovarian steroids regulate sleep-wake behavior in adult female rats and specifically determine if the preoptic area is the locus of these effects. Using an exogenous hormone replacement model that mimics the estrous cycle levels and timing of E2 and progesterone, we first tested whether E2 alone is sufficient to induce changes in sleep-wake behavior and sleep homeostasis or if progesterone has additional actions. Second, using pharmacological manipulations of local estrogenic signaling in the preoptic area sleep

nuclei, with ER antagonism and local E2 infusion, we investigated whether E2 is necessary and sufficient to induce changes in sleep-wake behavior and homeostasis. These experiments test the hypothesis that (1) E2 acting alone at the MnPN is (2) necessary and (3) sufficient to induce the changes in sleep seen with high hormone levels in cycling female rodents.

B. Materials and Methods

1. Steroid Treatments.

For experiment 1, all animals were administered 50uL of sesame oil on Day 1. Animals were subsequently administered 5 μg 17-β-estradiol benzoate in 50uL sesame oil (E2; Sigma-Aldrich, St. Louis, MO) on Day 2, and 10 μg E2 in 100uL sesame oil 24 h later on Day 3, or equivalent amounts (50uL/100uL) of sesame oil vehicle, through subcutaneous flank injections. On Day 4, animals received a dose of 500mg progesterone in 50μL sesame oil vehicle, or sesame oil vehicle control. Experiment 2 follows the same timing paradigm with the omission of the Day 4 progesterone injection. For experiment 3, 5ug cyclodextrin-encapsulated E2 in 5uL saline (Sigma-Aldrich), or 5uL free cyclodextrin vehicle (Sigma-Aldrich), was infused directly into the MnPN in each of three successive injections 24 hours apart. Experimental manipulation and sleep data collection was performed at times from 4 to 36 hours after the second hormonal injection (see specific experiments below).

2. Drugs and Infusion Paradigm.

Animals in experiment 1 comprised a single group that all received ovariectomy and identical hormone replacement as described above, with each animal's individual baseline serving as a control. Animals in experiment 2 were randomly assigned into either the

vehicle (VEH; 0.25% dimethyl sulfoxide (DMSO) in sterile saline) or ICI (50ng in 0.25% DMSO in sterile saline; Sigma-Aldrich) infusion groups, and reversed the following week for a second round of infusions. For targeted infusions to the VLPO, the dummy stylet was removed and replaced with a 33-gauge micro-infusion needle, which extends 2.0mm below the tip of the guide cannula. For targeted infusions to the MnPN, the dummy stylet was removed and replaced with a 33-gauge micro-infusion needle (Plastics One), which extends 0.5mm below the tip of the guide cannula. The needle was connected to a Hamilton 1705 RNR 50ul syringe (Hamilton, Reno, NV) via polyethylene tubing. A BASi Bee pump and Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, IN) was used to deliver ICI or VEH at a rate of 0.1μl/min. Following infusion, the needle remained in place for 5 minutes to ensure diffusion. ICI or VEH was infused 3 times per injection: (i) 6-12h prior to, (ii) 30 minutes prior to and (iii) 12h after injections (Fig. 16). Similarly, animals in experiment 3 received targeted infusions to the MnPN of cyclodextrin-encapsulated E2 or cyclodextrin vehicle; the dummy stylet was removed and replaced with a 33-gauge micro-infusion needle (Plastics One), which extends 0.5mm below the tip of the guide cannula. The needle was connected to a syringe and controller as described above. The setup was used to deliver cyclodextrin-encapsulated E2 or cyclodextrin vehicle at a rate of 0.1μl/min. Following infusion, the needle remained in place for 5 minutes to ensure diffusion.

3. Cannula Placement Verification

At the end of each experiment, animals were overdosed with a ketamine/ acepromazine mix before being transcardially perfused with 0.9% saline + 2% sodium nitrite followed by 4% paraformaldehyde in 0.05M KPBS. The brains were removed and

post-fixed overnight in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose in KPBS, frozen on dry ice, and stored at -80°C. Each brain was cut on a cryostat along the coronal plane at 30µm thick into 4 series and stored in an ethylene glycol-based storage solution at -20°C. Sections in each series are separated by 120µm.

Sections corresponding to the VLPO and MnPN from one series were mounted on 2% gelatin-coated slides. The slides were processed for cresyl violet (0.1% solution; cresyl violet acetate, Sigma-Aldrich) staining to examine cannula placement. VLPO hits were counted as placement within sections 32-36 of the brain atlas[198] and MnPN hits were counted as placement within sections 33-35. For experiment 2 in the VLPO, one animal was a miss and excluded from the study, while 3 animals were euthanized prior to completion and removed from the study. For all MnPN cannulations, animals with cannula placement outside of this area were removed from analysis; 3 animals were removed. There was 1 animal in experiment 2 whose cannula placement was a miss but remained in the analysis; this animal was infused with VEH and her behavior was not different from hits.

4. Statistical Analysis.

All data are represented as mean ± SEM. Two-way, repeated measure ANOVAs followed by Sidak post-hoc tests were run for each vigilance state to determine if direct VLPO and MnPN infusions significantly altered E2 effects on sleep-wake. Since this was a within-animal study, systemic injection (oil vs. E2) was the repeated factor and infusion (VEH vs. ICI) was the independent factor. An *a priori* comparison of interest was between the VEH and ICI infused E2 days of analysis. We ran an unpaired t-test to compare means on the E2 day between VEH and ICI infused animals. T-tests were used to compare E2 and VEH MnPN infusions and two-way, repeated measure ANOVAs followed by Sidak post-

hoc tests were run for analysis across the phase in 1h bins. Mann-Whitney U nonparametric tests were run to analyze differences between mean percent changes of each vigilance state. All statistical tests were conducted using the Graph Pad Prism program (San Diego, CA) on a PC. In all figures (*) denotes significance at $p<.05$, (**) denotes significance at $p<.01$, (***) denotes significance at $p<.001$, and (****) denotes significance at $p<.0001$.

C. Results

Experiments 1 and 2 were originally performed by, and data for Experiment 2 collected by, Danielle M. Cusmano, PhD. Except where noted data has been reinterpreted by PCS.

1. Estradiol is the Ovarian Steroid that Predominantly Influences Sleep-Wake Behavior in the Adult Female Rat.

Our laboratory and others have demonstrated that exogenous E2 administration, which mimics the levels and timing of the fluctuations in endogenous hormones, markedly reduces time spent in NREM and REM sleep with a concomitant increase in the time spent in wake.[6-8,80,110-111] However, these previous findings have only analyzed the 24 hours after the last injection of E2. To further explore and establish this model of ovariectomy followed by hormone replacement, which mimics the natural rise of E2 to peak proestrus level[80] and also recapitulates the sleep patterns of intact females,[7] we recorded and analyzed sleep across the treatment paradigm. We ovariectomized (OVX) female rodents and replaced E2 and progesterone globally through subcutaneous injection, in a cycle formulated to mimic endogenous hormone steroid levels. This replacement paradigm consists of an oil dose on Day 1 designed to mimic metestrus, a low 5ug E2 dose on Day 2 designed to mimic diestrus, and a high 10ug E2 dose on Day 3 designed to mimic proestrus.[80] (See “Steroid Treatments” in section II-B-3.) The advantage of this established

model (ovariectomy; OVX + exogenous E2 replacement that mimics the gradual natural rise of E2 to peak proestrus levels) is the standardization and reproducibility of circulating E2 levels on specific recording days. Following the second E2 treatment, the animals were divided into two groups and administered a physiological dose of progesterone (1mg; based on our established findings[7] or vehicle (referred to as Post E2) on Day 4. (Fig. 7).

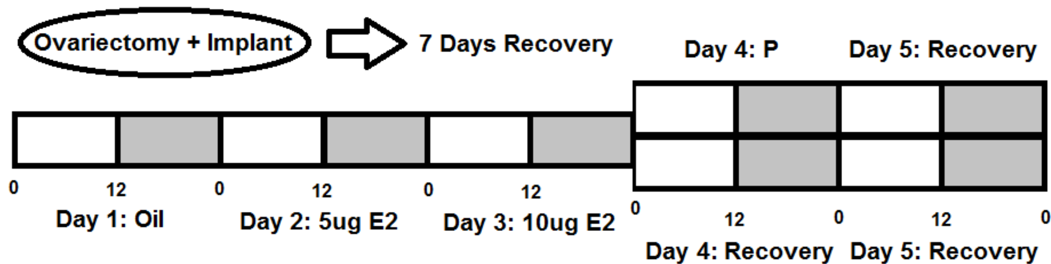


Fig. 7. Timeline of Hormonal Recapitulation Experiment. Ovariectomized Female Sprague-Dawley Rats (n=17) were administered an oil injection on Day 1, 5ug of E2 on Day 2, and 10ug of E2 on Day 3. On Day 4, the animals were split into two groups, with one group being administered progesterone to mimic the estrous hormone milieu and one group being administered vehicle to solely examine solely the effect of E2. Sleep times were measured using EEG/EMG telemetry (DSI Inc. St. Paul, Minn.)

a. Proestrus-Level Estradiol is Sufficient to Suppress Sleep

As anticipated, in the dark phase, E2 significantly increased the time spent in wake at the expense of NREM. This change was present on the day of high E2 administration. (Fig. 8-9-10) compared to the oil baseline.

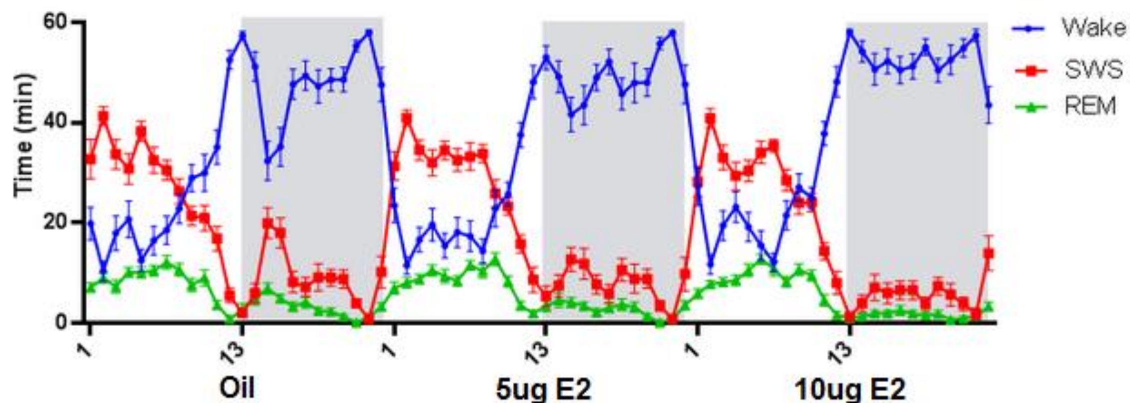


Fig. 8. Proestrus-level E2 Suppresses Sleep and Increases Wake. Using a within animal design, we recorded EEG/EMG data from OVX adult rats treated with our standard dosing

paradigm of 2 injections of E2 24 hours apart. On the day of high-dose E2 administration (Day 3), mimicking proestrus hormone levels, there is an increase in wake time and decrease in slow wave sleep time. This sleep change mimics the change in sleep on proestrus in naturally cycling rodents. (Repeated measure ANOVA; Wake main effect of treatment: $F(2,29)=13.37$, $p<0.0001$); (Repeated measure ANOVA; NREM main effect of treatment: $F(2,29)=14.15$, $p<0.0001$).

Conversely to the changes in wake (Fig. 9A), there was a significant decrease in NREM sleep time in the dark phase on the day of high E2 administration (Fig. 9B). E2 administration also significantly decreased REM sleep. (Fig. 9C)

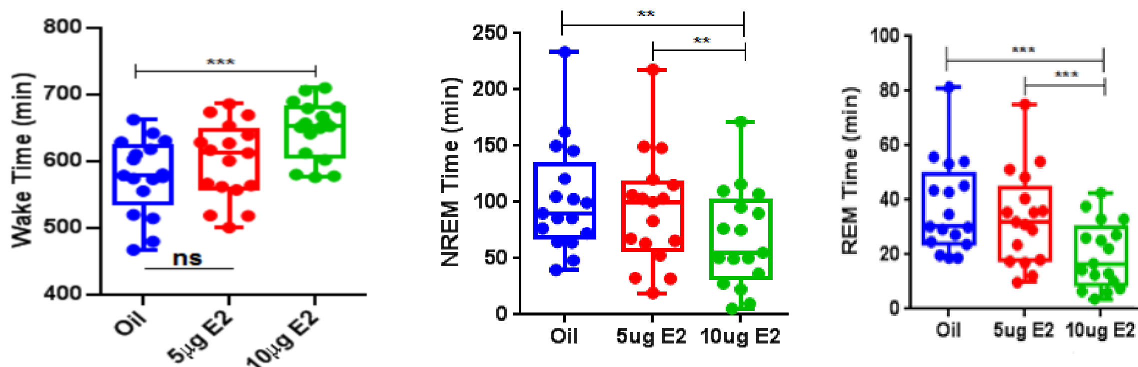


Fig. 9 A-B-C. E2 Increases Total Dark Phase Wake Time and Decreases Total NREM and REM Sleep Time. **(A)** In the dark phase, animals showed a significant increase in wake time relative to oil on the day of high-dose E2 treatment ($p=.0005$). The low-dose E2 treatment did not have a significant effect on wake, while the progesterone-treated animals did not show a significant difference in wake from their E2-only treated counterparts on the same day. (Repeated measure ANOVA; Wake main effect of treatment: $F(2,29)=13.37$, $p<0.0001$). **(B)** The high-dose E2 showed a significant decrease in NREM sleep time in the dark phase compared to both oil ($p<.01$) and low dose E2 ($p<.01$). (Repeated measure ANOVA; NREM main effect of treatment: $F(2,29)=14.15$, $p<0.0001$) **(C)** The high-dose E2 showed a significant decrease in REM sleep time in the dark phase compared to both oil ($p<.001$) and low dose E2 ($p<.001$). (Repeated measure ANOVA; REM main effect of treatment: $F(2,29)=15.43$, $p<0.0001$)

It is interesting to note that on the day analogous to proestrus (Post E2), E2 treatment abolished the mid-phase siesta by markedly increasing wake at ZT 14-16 compared to the baseline and low E2 days (Fig. 10). This effect was not present in the light phase, with no significant change in light phase sleep time noted across any of the treatment days. (Fig. 11)

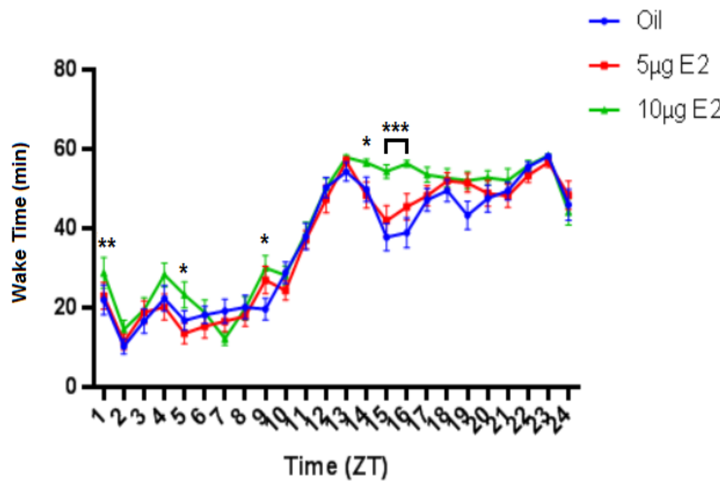


Fig. 10. E2-mediated Sleep Suppression is Most Prevalent in the Early Dark Phase. Analysis of the sleep time by hour across the treatment days, shows that wake time is not significantly different between the oil and low dose E2 at any treatment time. However, the high-dose E2 treatment showed a significantly higher wake time at six hourly time points, with the effect being particularly pronounced in the early dark phase (ZT-14-16) (Repeated Measure 2-way ANOVA; Main effect of treatment $F(2,48)=46.50$; $p<0.0001$. Sidak's multiple comparison test, Oil vs. 10ug E2, ZT 1 $p<.01$, ZT5 $p<.05$, ZT 9 $p<.05$, ZT 14 $p<.05$, ZT 15 $p<.001$, ZT 16 $p<.001$)

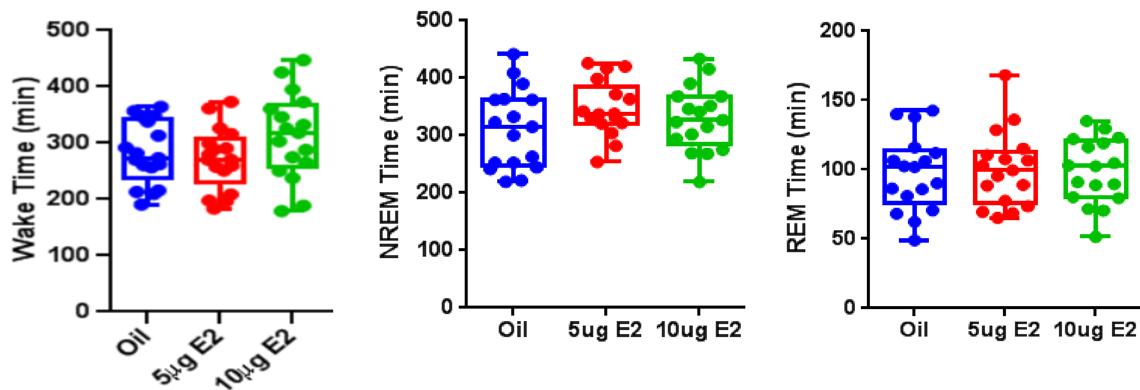


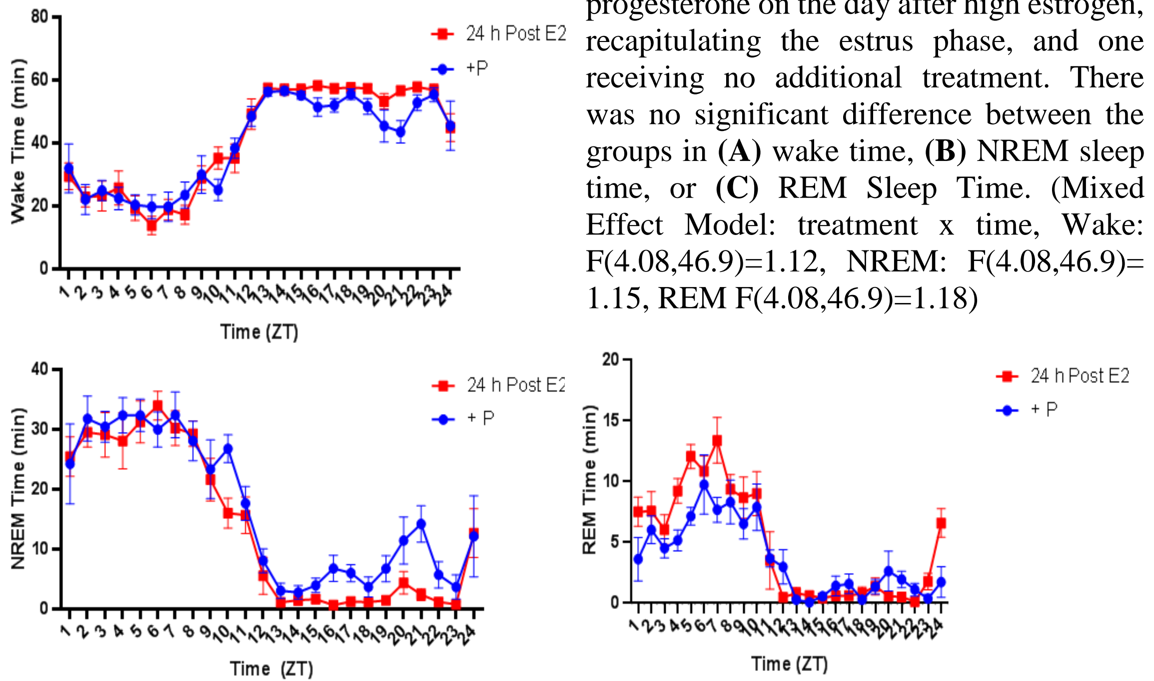
Fig. 11 A-B-C. E2 does not Affect Total Sleep or Wake Time in the Light Phase. There is no significant difference in **(A)** wake time, **(B)** NREM sleep duration, or **(C)** REM Sleep Duration across any treatment.

b. Progesterone Has No Significant Additional Effect on Sleep-Wake States

After showing the effects of E2 alone, a question remained over whether progesterone, which also rises on the afternoon of proestrus in natural cycling females, is influencing sleep and wake. Thus, to further validate our model, following the second E2 treatment, on Day 4 the animals were divided into two groups and administered a physiological dose of progesterone (P; 1mg, which is a dose relevant to endogenous proestrus levels)[199] or vehicle (referred to as Post E2). Moreover, we also analyzed sleep

times with and without progesterone. We found that progesterone had no significant effect on sleep-wake states, either wake, NREM, or REM, when compared to the analogous Day 4 (Post E2) day (Fig. 12A-B-C).

Fig. 12 A-B-C. Progesterone does not Affect Sleep Times. To compare for the effect of Progesterone, we split the cohort into two treatment groups on Day 4, one receiving progesterone on the day after high estrogen, recapitulating the estrus phase, and one receiving no additional treatment. There was no significant difference between the groups in **(A)** wake time, **(B)** NREM sleep time, or **(C)** REM Sleep Time. (Mixed Effect Model: treatment x time, Wake: F(4.08,46.9)=1.12, NREM: F(4.08,46.9)= 1.15, REM F(4.08,46.9)=1.18)



Furthermore, we also analyzed NREM delta (0-4 Hz) power through Fourier transformation of the EEG signal, a widely used[200-201] measure of the depth of homeostatic sleep, both with and without progesterone. When normalizing delta power to each animal's baseline oil day (Day 1), we found no significant change in the relative delta power difference between progesterone-treated and untreated animals. However, in both groups, there was a decrease relative to oil baseline in the E2-treated animals (Fig. 13). Thus, these findings validate the ovariectomy + exogenous E2 (alone) model as a reliable experimental system that is amenable to local manipulation of sleep-active nuclei to more directly test how estrogens modulate the sleep-circuits and elicit changes in sleep behavior, and show

that global E2 action alone is sufficient to recapitulate sleep changes in naturally cycling rodents.

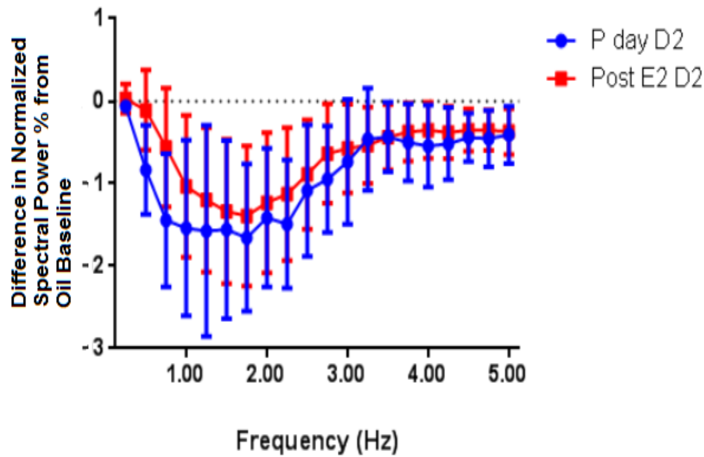


Fig. 13. Progesterone Produces No Change in EEG Spectral Power. We compared power spectra of the progesterone and non-progesterone treated groups on Day 4 relative to their own Day 1 oil baseline. In both groups, we see a decrease in spectral power on the day of progesterone treatment, with no significant difference between the progesterone treated and E2 only groups.

c. Estradiol Decreases NREM-SWA Spectral Power

To investigate these EEG power spectrum findings (Fig. 13) further, we examined the differences in EEG power between oil-treated and post-E2/E2+P animals, comparing each animal's Day 1 and Day 4 readings. The power frequency distribution of dark phase NREM-SWA from the females used in the progesterone experiment was compared between Oil versus the day post-E2, which represents the period of the greatest NREM sleep loss following E2 administration, with or without progesterone. We found that there was no significant change in power across all sleep states during either the first half (Fig. 14A) or second half (Fig. 14B) of the light phase. However, during the dark phase, significant changes were observed. Given the significant decrease in dark phase NREM sleep (~40%), we expected the NREM-SWA frequency distribution in the 0.5-4.0 Hz bands to be significantly greater following E2 treatment. However, E2-treated animals, both with and without progesterone, showed a significant *decrease* in EEG power in the low frequency ranges, particularly in the delta band. (Fig. 14C) Comparisons of the normalized

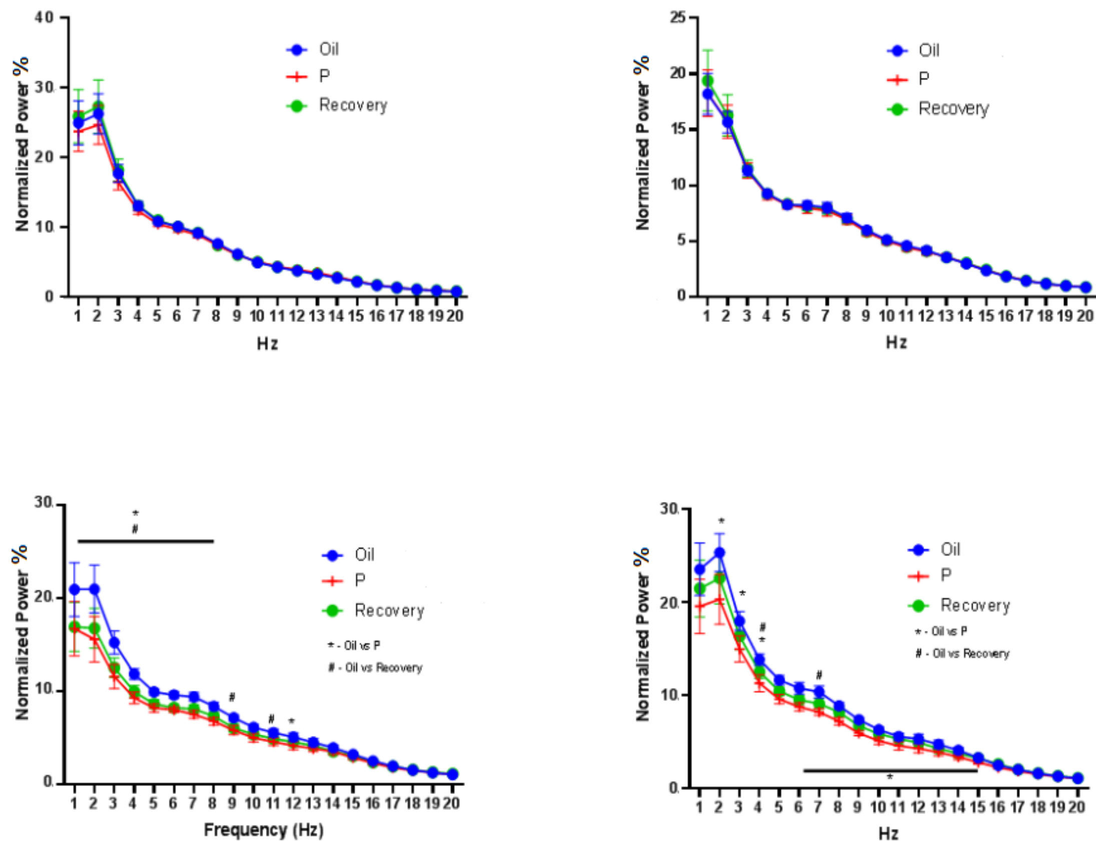


Fig. 14 A-B-C-D. E2 Decreases Spectral Power in the Dark Phase, Particularly in the Delta Band. We examined the spectral power across all animals on Day 1, the day of oil treatment (Oil), Day 4, the day of progesterone treatment (P) following E2, or the following Day 5 (Recovery). (See Fig. 7) There was no significant change in spectral power at any frequency in the light phase, either the ZT 0-6 early portion **(A)** or the ZT 6-12 later portion **(B)**. In the dark phase, however, there was a pronounced decrease in normalized power at low frequencies, particularly in the delta and theta ranges in the first half of the dark phase ZT 12-18 **(C)**, when sleep times are most affected by E2. These low power ranges have been shown to be important for homeostatically restorative sleep. (REML Mixed-Effects model with multiple comparisons, main effect of hormone, $F(19, 200) = 105.9$, $p<.001$, interaction of hormone X time, $F(57, 580) = 1.484$, $p<.05$) In the second half of the dark phase ZT18-0 **(D)** there was a significant decrease in the Theta and Alpha frequency ranges as well as the Delta. (REML Mixed-Effects model with multiple comparisons, main effect of hormone, $F(19, 200) = 141.0$, $p<.0001$, interaction of hormone X time, $F(57, 580) = 1.901$, $p<.001$)

percent of total power spectral distribution revealed that E2 significantly decreased the dark phase power of the 1-2.5 Hz bands, which typically represent the highest level of cortical synchronization and thus high-quality sleep, suggesting a decreased level of deep,

homeostatically restorative sleep. In the second half of the light phase, the effect of lowered EEG power with E2 was less-pronounced, (Fig. 14D) but appeared to be present over a broader range of frequencies, including in the theta band. These results suggest that the decrease in NREM sleep time in E2 also manifests as a decreased level of deep, homeostatically restorative sleep, and that E2 may attenuate the build-up of SWA under normal physiological conditions.

2. Estrogen Receptor Antagonist Action at the MnPN but not the VLPO of Adult OVX Females Attenuates Estradiol Mediated Suppression of Sleep.

a. ER Alpha Expression is Present at High Levels in the Female MnPN but not the VLPO.

Building on those results, we next attempted to determine if the POA circuitry is necessary to drive these effects. Over the past decade, numerous studies using various techniques have convincingly demonstrated that neurons in the VLPO and the MnPN are involved in sleep-regulatory mechanisms.[159-160] The VLPO and MnPN reportedly have complementary roles in the maintenance of sleep, as they both (i) have a predominant number of sleep-active cells (i.e., the number of Fos-ir neurons increases following episodes of sustained sleep but not sustained waking),[84,160] (ii) have a high concentration of neurons with elevated discharge rates during both NREM and REM sleep compared to waking (i.e. sleep-active discharge pattern),[172] and (iii) are thought to function to promote and sustain sleep by inhibiting key arousal centers via descending GABAergic (MnPN) and GABAergic/galaninergic (VLPO) projections [166,169-171] Here we investigate whether estrogen receptors were present in these sleep- associated nuclei. Immunocytochemistry

using polyclonal antibodies against ER alpha demonstrated a significantly greater population of ER alpha positive cells in the MnPN compared to the VLPO (Fig. 15). These findings show that the MnPN appears to be the major seat of E2 sensitivity in these active sleep circuits.

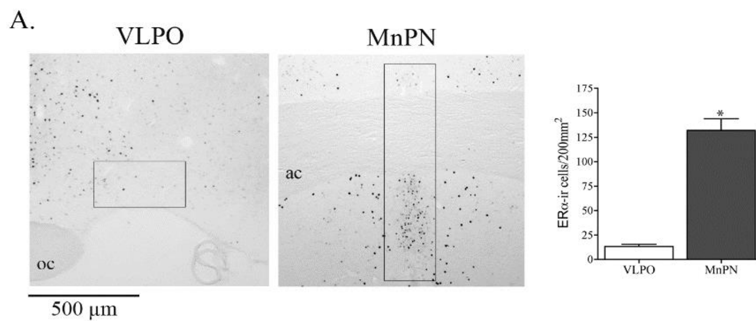


Fig. 15. (Figure from DMC) Estrogen Receptors are Highly Expressed in the Female MnPN. Staining for Estrogen Receptors (ER) show there is a high concentration in the MnPN but not the VLPO of females. (Student's *t*-test, *, $p<0.05$ vs. VEH).

b. ER Antagonist Infusion to the MnPN Partially Rescues E2-Mediated Sleep Suppression

Building on the presence of E2-receptors in these nuclei, we attempted to test if the MnPN is necessary to mediate E2 actions on NREM sleep. Using the same exogenous E2 replacement paradigm shown to produce effects on sleep, we then cannulated the VLPO and the MnPN and infused ICI-182-780 (ICI), an estrogen receptor (ER) antagonist. This study attempts to determine if estrogen receptor (ER) signaling is required in either region for E2 suppression of sleep. These experiments show that blocking ER signaling at the MnPN, but NOT the VLPO, is able to ameliorate E2-mediated sleep suppression.

Due to greater expression of ER alpha in the MnPN, we ran a preliminary cohort of infusion of the direct estrogen receptor antagonist ICI 182,780 (ICI) into the MnPN. OVX rats were hormonally replaced with E2 or Oil, using the same paradigm as in experiment 1. However, on days of E2 administration, an ER agonist, ICI, was also infused locally into the MnPN. (Fig. 16). Cannula targeting was confirmed by histology (Fig. 17).

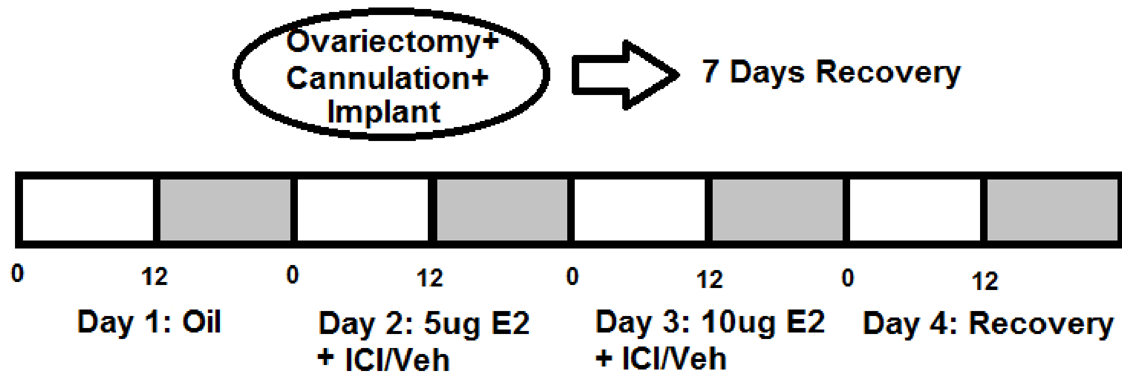


Fig. 16. Timeline of ER Antagonism Experiment. Ovariectomized Sprague-Dawley rats (n=15) were treated with hormone replacement of either E2 or Oil using the same paradigm as in fig. 1-3 (5ug Day 1, 10ug Day 2) and cannulated to the MnPN. A subset of the animals (n=8) were treated as well with the Estrogen Receptor antagonist ICI through direct local infusion to the MnPN and the others (n=7) were treated with vehicle. Sleep behavior measured with EEG/EMG telemetry (DSI Inc., St. Paul MN).

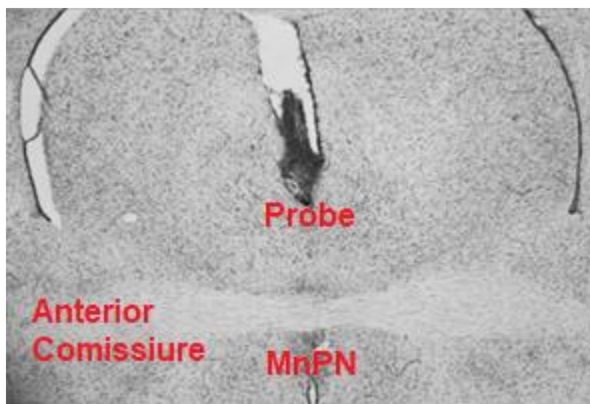


Fig. 17. Representative Image of MnPN Probe Placement. Probe placement to the MnPN was confirmed histologically.

The effects of ICI in the MnPN on E2 suppression of sleep were moderate to high for wake (d = 0.67), total sleep (d = 0.67), NREM sleep (d = 0.5), and REM sleep (d = 1.36), prompting further investigation. In all groups, systemic E2 significantly modulated sleep-wake behavior; there was a main effect of E2 treatment for wake, total sleep, NREM sleep, and REM sleep during the dark phase. E2 treatment increased the time spent in wake and decreased sleep, both NREM sleep and REM sleep during the dark phase. In this study, pairwise comparisons of VEH infused animals given oil then E2 revealed that E2 treatment increased wake duration and decreased total sleep and REM

sleep. Direct infusion of ICI into the MnPN significantly attenuated these effects during the dark phase, for both Wake and NREM sleep (Fig. 18A-B).

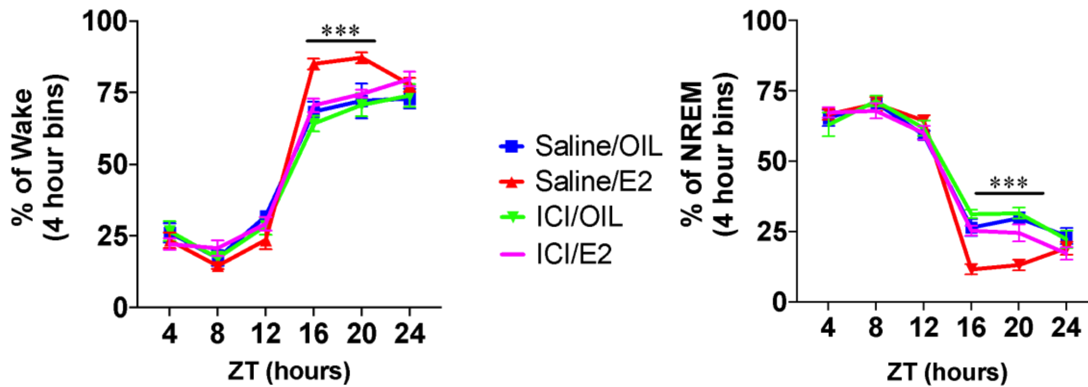


Fig. 18 A-B. ER Antagonist ICI Reduces Wake Time and increases NREM Sleep Versus E2 Replacement. (A) Injection of ICI against a background of E2 treatment reduced wake time across much of the dark phase, with statistically significant decreases from ZT 12-16 ($p<.001$) and ZT16-20 ($p<.001$). There was no significant difference between ICI-treated and ICI-untreated animals without E2 replacement. **(B)** Injection of ICI against a background of E2 treatment increased NREM time across much of the dark phase, with statistically significant increases from ZT 12-16 ($p<.001$) and ZT16-20 ($p<.001$). There was no significant difference between ICI-treated and ICI-untreated animals without E2 replacement. Main effect of E2 treatment for wake, ($F_{1,12} = 53.48$, $p < 0.001$)/ ($t_5 = 2.56$, $p = 0.05$), total sleep ($F_{1,12} = 53.48$, $p < 0.001$)/ ($t_5 = 2.68$, $p = 0.04$), NREM sleep ($F_{1,12} = 39.93$, $p < 0.001$) and REM sleep ($F_{1,12} = 57.03$, $p < 0.001$)/ ($t_5 = 2.78$, $p = 0.04$) during the dark phase.

Animals who received direct infusions of ICI into the MnPN acquire about 47 minutes less wake than VEH and about 37 more minutes of NREM sleep and 10 more minutes of REM sleep (Fig. 19) during the dark phase. The percent change in wakefulness induced by E2 was not significantly different between VEH and ICI infusion groups. However, the percent changes in total sleep, NREM sleep, and REM sleep induced by E2 were significantly attenuated by ICI during the dark phase. As anticipated, the saline/E2 treated animals had significantly increased wake and reduced NREM sleep during the dark phase following the last injections. However, treatment with ICI (ICI/E2) blocked this E2 mediated effect, partially rescuing keeping NREM and REM sleep, and inhibiting

additional wake, to near baseline levels (Fig. 19). Keeping with the lack of significant effect of E2 in the light phase (Fig. 11), there was no effect of ICI on sleep times in the light phase (Fig. 20).

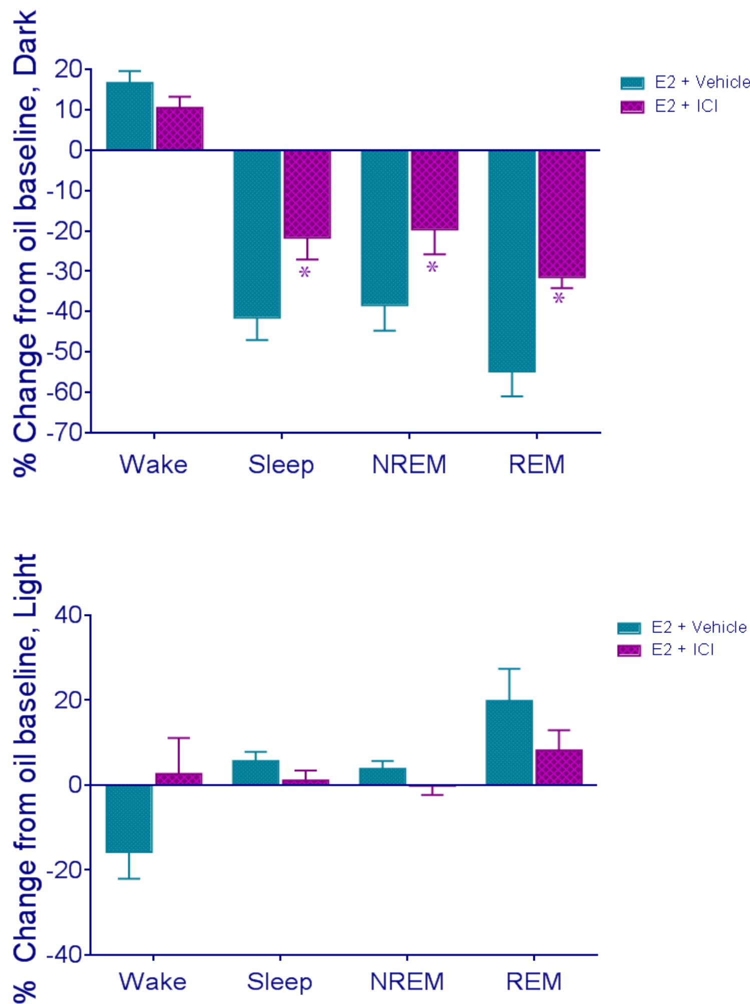


Fig. 19. ICI Treatment Partially Rescues E2-Mediated Dark Phase Sleep Suppression. We also compared total sleep time relative to each animal's oil baseline recording, both with E2 treatment and vehicle and E2 and ICI treatment. During the Dark Phase, ICI treatment partially rescued the E2-mediated decrease in sleep, both in NREM and REM phases, leading to an increase in sleep time relative to E2+Vehicle animals. (Dark phase two-way ANOVA; main effect of treatment, Wake: $F(3,26) = 9.157$; $p<0.0005$, NREM: $F(3,26) = 14.86$ $p<0.0001$) Wake ($t_{12} = 2.376$, $P = 0.04$) NREM sleep ($t_{12} = 2.158$, $p = 0.05$) REM sleep ($t_{12} = 2.518$, $p = 0.03$)

Fig. 20. ICI does not Significantly Change Sleep in the Light Phase. As E2 did not have significant effects on sleep in the light phase relative to oil baseline, there was no effect of ICI antagonism of ERs in in the light phase relative to E2 treatment alone.

c. ER Antagonist Infusion to the VLPO Does NOT Rescue Sleep Behavior

Conversely to the MnPN, in the VLPO, infusions of ICI in the presence of systemic E2 had no effect on NREM or wake. Findings suggest that ICI infusion into the VLPO does not attenuate E2 effects on wake and sleep (Fig. 21A-B). Therefore, our data

demonstrate that E2 acting directly in the MnPN and *not* the VLPO is necessary to attenuate NREM sleep, and that the inhibitory effect of E2 on sleep behavior is mediated by E2-expressing cells in the MnPN.

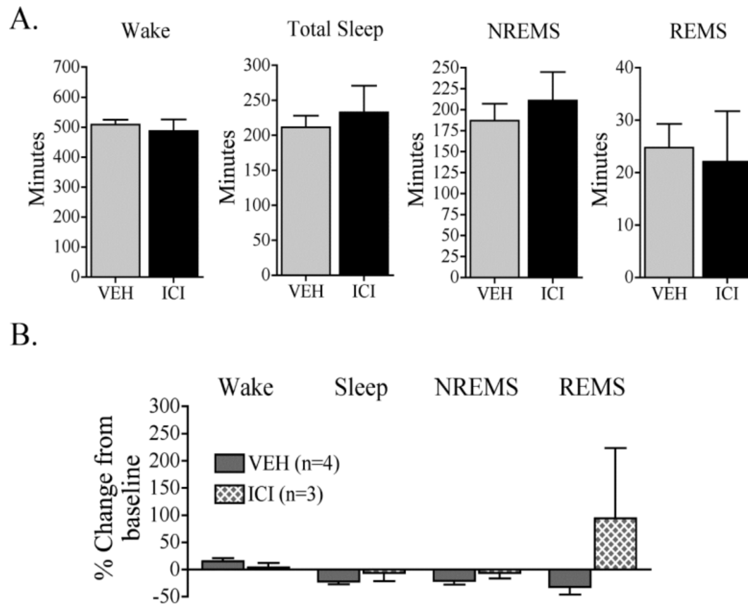


Fig. 21 A-B. (Figure from DMC) ICI Infusion to the VLPO does not Change E2-Driven Sleep Behavior. Unlike infusion into the MnPN, infusion of ICI into the VLPO did not affect wake time, total sleep time, or REM sleep time, either **(A)** in overall time or **(B)** change from oil control baseline. The effect size of the preliminary cohort for wake (d = 0.51), total sleep (d = 0.51), NREM sleep (d = 0.58), and REM sleep (d = 0.24) indicate any effect of ICI in the VLPO on E2 suppression of sleep was small to moderate.

3. Direct Infusion of Estradiol into the MnPN Increases Wake and Suppresses Sleep. Finally, we investigated whether E2 is sufficient to suppress sleep. To test this aspect of the signaling, we replaced the global subcutaneous administration of E2 with direct local infusion into the MnPN, to determine if E2 acting specifically at that nucleus is sufficient to reduce sleep. These studies will address whether estrogen receptor signaling in the VLPO and/or the MnPN is the key site of action for the E2-mediated suppression of sleep in metrics of both necessity and sufficiency. OVX female rats were implanted with EEG/EMG telemeters and guide cannula to the MnPN. After recovery, animals were infused with 3 doses of cyclodextrin- encapsulated E2, a water soluble form of E2, or equivalent amount of free cyclodextrin vehicle. This treatment was performed over 3 successive days at ZT18. (Fig. 22).

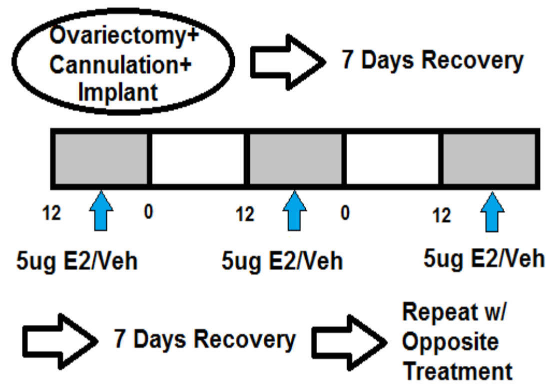


Fig. 22. Timeline of Direct E2 Infusion Experiment. Female Sprague-Dawley rats (n=9) were ovariectomized and implanted with EEG/EMG telemeters and guide cannula to the MnPN. After recovery, animals were infused at ZT18 with 5ug cyclodextrin- encapsulated E2 in 5uL sterile saline, or 5ug cylodextrin vehicle in 5uL sterile saline. The same treatment was repeated for 3 successive days. After a 4-day washout, animals were subjected to the other treatment. Sleep architecture was quantified.

The significant differences in wake and NREM sleep were observed only in the light phase following the second injection. E2 infusion showed sleep suppression during the light phase on the second day of treatment, which significantly increased wake (Fig. 23) and decreased NREM sleep (Fig. 24) over the entirety of the light phase. There was no change in REM sleep (Fig. 25). In agreement with previous results showing a lack of hormone effects on sleep in males,[8] ICI treatment showed no effect in males.

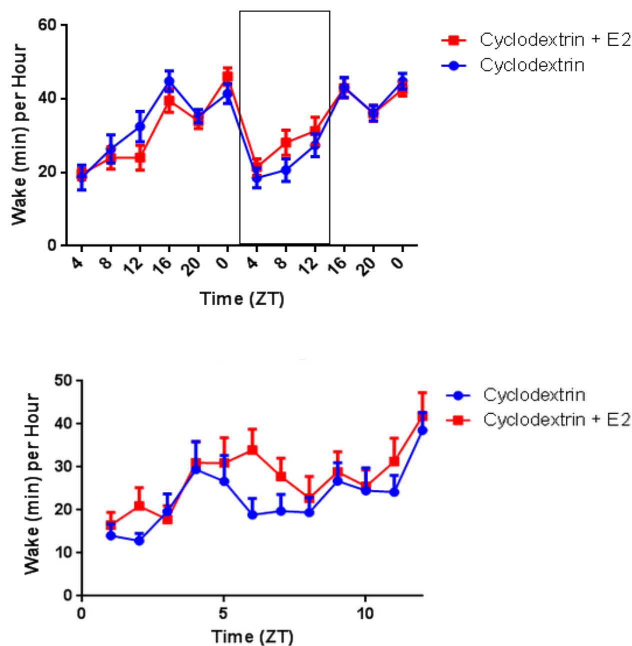
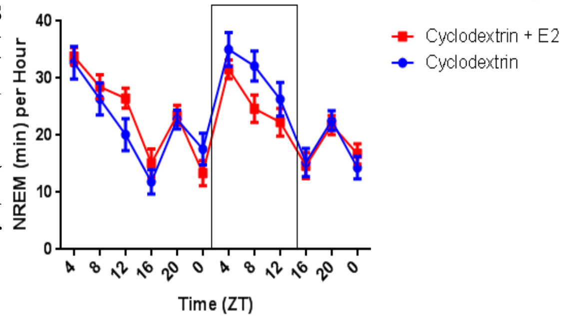
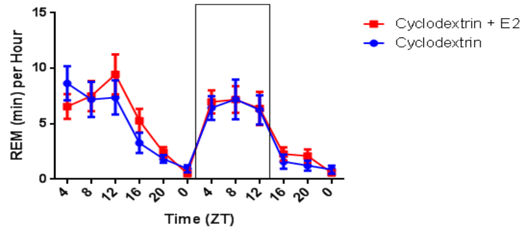
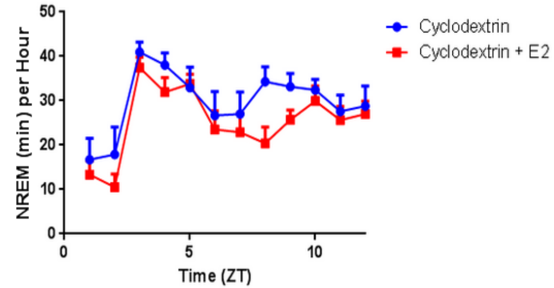


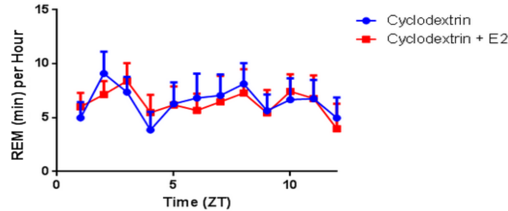
Fig. 23 A-B. Direct E2 Infusion Increases Dark Phase Wake. E2-treated animals showed an increase in wake time in the 2nd light phase (p=.03, two-way ANOVA, main effect from ZT0-12 on treatment day 2). Lower Panel is increased detail of boxed region.

Fig. 24 A-B.> Direct E2 Infusion Tends Toward a Decrease in Dark Phase NREM Sleep. There was no significant difference in NREM sleep time between the two groups, though the E2-treated animals did show a trend (p=.10 main effect, two-way ANOVA ZT 0-12 day 2 of treatment) toward lower sleep time in the second light phase.









<Fig. 25 A-B. Direct E2 Infusion Has No Significant Effect on REM Sleep. There was no significant difference in REM sleep time between the two groups.

a. Estradiol Infusion Decreases NREM-SWA

We analyzed EEG spectral power in the second light phase with and without E2. While there was no significant change in NREM Delta Power over the entirety of the experiment, analysis of the second light phase showed decreases in delta power across many time points, particularly in the early part of the period (Fig. 26)

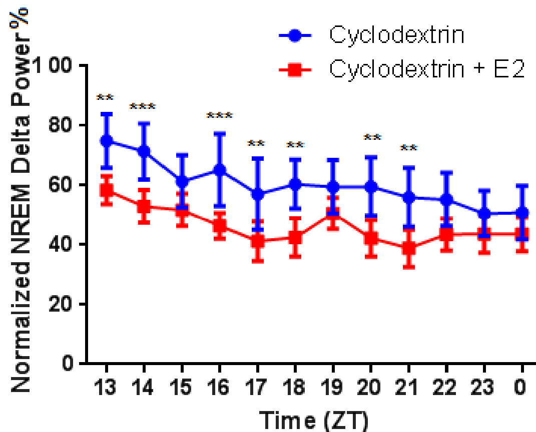
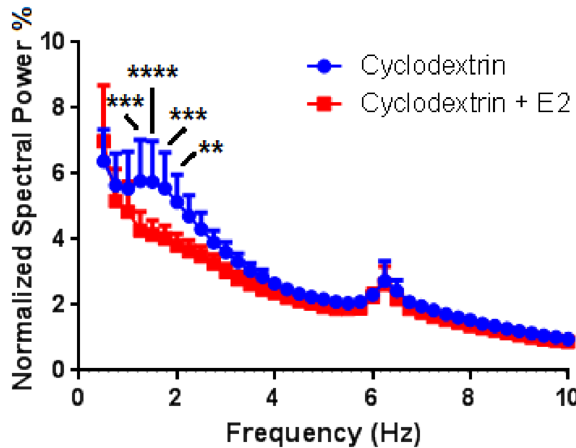


Fig. 26. E2 Infusion Decreases NREM Delta Power. We analyzed the spectral power with and without E2. NREM Delta Power from E2-treated animals shows a significant decrease in several time points in the second light phase relative to oil controls. (Repeated Measures two-way ANOVA, main effect of hormone, $F(1, 9) = 3.228$ $P = 0.1059$ post-hoc Sidak's multiple comparison test, ZT 13 $p<.01$, ZT 14 $p<.001$, ZT 16 $p<.001$, ZT 17-18 $p<.01$, ZT 20-21 $p<.01$)

Further spectral analysis of this period shows that the effect is concentrated in the lowest frequencies of the delta band (below 2Hz), showing a decrease in the most coordinated brain waves that signify deep homeostatic sleep. (Fig. 27) Together, these findings strongly suggest that the MnPN is a direct mediator of E2 actions on sleep, and that E2 action at the MnPN is both necessary and sufficient for estrogenic effects on sleep.

Fig. 27. Direct Infusion of E2 Changes Power Most Significantly in the Low Delta Band. Spectral Fourier analysis revealed the decrease in delta power was localized to a particular portion of the low delta band, with the difference between E2 and vehicle significant at the 1.25 ($p<.001$), 1.5 ($p<.0001$), 1.75 ($p<.001$), and 2 ($p<.01$) Hz bands. (Repeated Measures two-way ANOVA, main effect of hormone, $F(1, 9) = 4.129$, post hoc Sidak's multiple comparison test, 1.25Hz band $p<.001$, 1.5Hz band $p<.0001$, 1.75Hz band $p<.001$, 2Hz band $p<.01$).



D. Discussion

Previous research studies using rodent models describe the changes in sleep across the female estrous cycle and following gonadectomy.[7-8,76,80] Studies consistently reported that E2 suppresses NREM sleep and REM sleep in females, while changes in gonadal steroids cause little to no change in sleep in males. Here, we sought to address the mechanism by which proestrus levels of cycling ovarian steroids suppress sleep in females. We show that after hormone replacement of proestrus levels of E2, the suppression of sleep by endogenous hormones may be recapitulated. We further show that this suppression is due to the high levels of E2 alone, and that progesterone, the other major circulating ovarian steroid, did not have a significant impact on sleep behavior. Extending these findings, we found that E2 has direct actions within the sleep-active POA, specifically in the MnPN,

which contains estrogen receptors (ERs). Antagonizing of ERs in the MnPN, but not the VLPO, attenuated the E2-mediated suppression of both NREM and REM sleep. We finally found that, in addition to E2 actions at the MnPN being necessary for E2 suppression of sleep, it is also sufficient, as the direct infusion of E2 into the MnPN suppressed sleep with no other intervention. Based on our findings, we predict that proestrus levels of E2 alone, acting at the MnPN, mediate sex-hormone driven suppression of sleep in female rats.

From our findings, we further predict that E2 is both necessary and sufficient to reduce the activation of MnPN sleep active cells, thereby releasing the inhibitory tone on downstream targets. The MnPN contains GABAergic sleep-active projection neurons, which innervate the lateral hypothalamus and multiple brainstem nuclei.[166] GABAergic MnPN neurons have direct inhibitory control over the orexinergic neurons in the perifornical area/ lateral hypothalamus.[167] These orexinergic neurons are a key source of arousal signaling, suggesting a sleep-promoting mechanism of the MnPN. Since ICI had little to no effect within the VLPO, while E2 in the MnPN was sufficient to induce changes, E2 is most likely acting predominantly on the MnPN and *not* acting directly on the neural circuits of the VLPO. However, as the MnPN also innervates the VLPO,[168] a decrease in MnPN activation by E2 may elicit a similar decrease downstream in the VLPO. Additionally, the sex difference in MnPN ERα expression may account for the difference in sensitivity of males and females to the suppressive effects of E2 on sleep.[8]

1. Potential Molecular and Neurological System Mechanisms of Estradiol Effects on Sleep

Beyond the question of a site of action, the question of how, in terms of molecular and neurological mechanism, E2 may be meditating sleep effects is an important one. Two

distinct systems govern aspects of sleep regulation, the circadian wake system and the homeostatic sleep pressure system, which operate in concert to generate an overall sleep pressure that is responsive to both the animal's intrinsic homeostatic needs as well as external factors such as the light-dark cycle. The homeostatic sleep pressure system, which governs the amount of sleep needed after a given period of wake to maintain homeostasis, independent of circadian factors, is thought to utilize both the VLPO[159] and MnPN[5] as key originators of this pathway. The VLPO and MnPN send GABAergic projections to key mediators of the wake state, including nuclei in the lateral hypothalamus governing the orexinergic wake system.[5] Additionally, the VLPO and MnPN have been shown as sites of sensitivity to adenosine, an important mediator of homeostatic sleep pressure.[151] Further exploration of these molecular and neurological pathways could provide greater insight into precisely how E2 is affecting sleep need and behavior.

E. Conclusion

Rodents provide a model system for studying the mechanism underlying the sensitivity of the sleep circuitry and behavior to E2. Such a model is highly significant in the identification of neuronal targets for E2 within the sleep circuitry. Here, we describe the key role of E2 alone in modulating sleep behavior, as well as provide first clear evidence of a direct role for E2 in a sleep-active nucleus. The identification of the MnPN as a direct site of E2 action, showing that it is both necessary AND sufficient for induction of estrogenic effects on sleep, now allows for more mechanistic research to determine how E2 is suppressing sleep in females. Understanding the circuits that E2 can act on to regulate sleep may enable better drug development and treatment of sleep disorders in the clinical population.

III. Estradiol Influences Adenosinergic Homeostatic Sleep Need in Adult Female Rats.

A. Introduction

The ventrolateral preoptic area (VLPO) and the median preoptic nucleus (MnPN) have been implicated in sensing homeostatic sleep need,[159] which is defined as the required amount of sleep in a given time period that compensates for the prior period of time spent in wake to maintain homeostasis.[140] Moreover, previous findings suggest sleep-active neurons in the VLPO are sensitive to fluctuations in ovarian steroids.[7] E2 replacement following ovariectomy reduces neuronal activation of VLPO sleep active neurons as well as mRNA expression and protein levels of lipocalin-type prostaglandin D synthase (L-PGDS), the synthesizing enzyme for the somnogen prostaglandin D2.[7,197] These data suggest that E2 action in the preoptic area nuclei may alter critical factors involved in sleep homeostasis. However, molecular mechanisms for how E2 is affecting the homeostatic sleep circuitry remain unelucidated.

Adenosine is a ubiquitous biological substance; though adenosine signaling has been shown to have multiple functions, somnogenic effects are a key property of the system. Adenosine has been shown to accumulate in the brain with increasing wake time and decrease in the sleep state.[149] Adenosine has been hypothesized as a dual marker and mediator of sleep pressure; however, behavioral and physiologic effects of interactions between E2 and adenosine have not yet been explored. Thus, we propose the premise that interactions between E2 and adenosine provide a mechanism for E2 effects on homeostatic sleep pressure.

In this study, we focus on determining the signal connection between E2, homeostatic sleep pressure, and sleep behavior. In particular, we strive to elucidate if there is a direct effect of E2 on adenosinergic sleep pressure. To accomplish this objective, we tested (1) if E2 reduces the homeostatic need for sleep by established behavioral assays of sleep behavior and architecture. We then (2) tested if E2 reduced the levels of adenosine, as a marker for the homeostatic need for sleep, by extracellular measures of adenosine content. Finally, (3) we attempted to pharmacologically stimulate the adenosinergic sleep pressure system and evaluate the effect of E2 to evaluate a direct connection between adenosinergic signaling and the effects of E2.

Overall, our data suggest that estrogen modulates the ability of adenosine to generate sleep pressure, and thus the effect of E2 on wake may be mediated through decoupling the molecular drivers of sleep pressure through the adenosinergic system from sleep behavior. These experiments test the overarching hypothesis that estrogenic modulation of sleep pressure requires the attenuation of adenosine signaling efficacy.

B. Materials and Methods

1. Hormone Treatments.

For experiments 1 and 2, animals were administered 5 μg 17-β-estradiol benzoate in 5uL sesame oil (E2; Sigma Aldrich, St. Louis, MO) followed by 10 μg E2 in 10uL sesame oil 24 h later, or equivalent amounts (5uL/10uL) of sesame oil vehicle, through subcutaneous flank injections. For experiment 3, the same timing paradigm was used with the doses being one-quarter the amount of E2 in an equivalent amount of oil vehicle (1.25ug/5uL Day 1, 2.5ug/10uL Day 2). Experimental manipulation and sleep data

collection was performed at times ranging from 4 to 36 hours after the second hormonal injection (see specific experiments below).

2. Cannula Placement Verification.

At the conclusions of experiments 2 and 3, brains were removed and submersion-fixed in a solution of 9% formalin in potassium phosphate buffered saline (kPBS; 0.5M, pH 7.4), at 4 °C, followed by cryoprotection in 30% sucrose in kPBS. After cryoprotection, the brains were frozen on dry ice and stored at -80 °C until processed for Neutral Red staining to examine cannula placement. Brains were sectioned (30 μm) in the coronal plane in a cryostat and dried on gelatin-subbed slides. Cannula placement was marked using the visually-determined tip point of the lesion created by cannula insertion. Such points falling within the Preoptic Area (Bregma -0.3mm to Bregma +0.4mm, within 1mm of median, and below the Anterior Commissure) for Experiment 2, or directly within the Median Preoptic Nucleus for Experiment 3, were counted as hits. Data from misses regardless of experimental group was not included in any analysis.

3. Statistics.

Results are expressed as means ± SEM. The distribution of data did not deviate significantly from normality. Two-way ANOVAs followed by Sidak t-test *post hoc* comparisons were used to analyze all data except in: Fig. 33 and 38, where a two-tailed student's *t* test was used to directly compare sleep times, and Fig. 40, where Kolmogorov-Smirnov tests were used to compare the cumulative probability distributions. All statistical tests were conducted using the Graph Pad Prism program (San Diego, CA) on a PC. In all figures (*) denotes significance at $p<.05$, (**) denotes significance at $p<.01$, (***) denotes significance at $p<.001$, and (****) denotes significance at $p<.0001$.

Experiment 1:

4. Sleep Deprivation.

To test the hypothesis that estrogenic effects on sleep are mediated by a modulation of homeostatic sleep need, in a within-animal design, sleep behavior was assessed across four conditions comprising a 2X2 matrix of Steroid (E2/oil) and Sleep Pressure (sleep deprivation/*ad libitum* sleep) conditions. Adult female Sprague-Dawley rats (n=11) were OVX, implanted with EEG/EMG transmitters and treated with E2 or oil vehicle (as described above in section III-B-3). A randomly selected cohort (n=3) was allowed sleep *ad libitum* for the duration of the light phase (referred to baseline sleep) A second randomly selected cohort of animals (n=3) was subjected to total sleep deprivation by gentle handling[202-203] for the first six hours of the light phase, followed by *ad libitum* recovery sleep for the remainder of the light phase. These cohorts serve to evaluate estrogenic effects under different sleep pressure conditions, both physiological and elevated. After 7 days of recovery, the animals were subjected to the opposite treatments in steroid treatment and deprivation interventions for a total of four interventions per animal. (See Fig. 28 for timeline). A third cohort of animals (n=5) was subjected to sequential interventions of both *ad libitum* sleep and gentle handling for six hours as in the other cohorts, but was also subjected, after 7 days of recovery, to sleep deprivation by gentle handling for the first three hours of the light phase, with *ad libitum* recovery sleep for the last nine hours of the light phase. This cohort received a total of six interventions per animal. Baseline and Recovery sleep-wake behavior was scored from the recorded EEG/EMG traces as described above. Time spent in each vigilant state, sleep architecture and NREM-SWA were assessed. The degree of sleep pressure was assessed by established behavioral

measures, including sleep time during recovery, NREM-SWA delta band power (0.5-4.5Hz), and sleep and wake bout lengths.[109]

Experiment 2:

5. Microdialysis.

To test whether E2 induced sleep changes were associated with altered levels of extracellular adenosine in the MnPN, adult female Sprague-Dawley rats were OVX, implanted with a guide cannula and treated with E2 or oil vehicle as described above. A subset of the animals were implanted with transmitters as described above. Approximately 6 hours after the Day 2 steroid injection, the dummy cannula was removed and replaced with a microdialysis probe with a 6kD membrane cutoff (SciPro Inc., Sanborn, N.Y., model #MAB-9.14.1). The 7mm probe extends 1mm beyond the end of the guide cannula. After at least 12 hours of probe acclimation (~ZT 22.5), the probes were attached via polyethylene tubing to a 25 µl Hamilton syringe (700 series, Hamilton, Reno, Nev.). The flowrate of the syringe was controlled by a BASi Bee pump attached to a Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, Ind.). The inserted dialysis probe was perfused at a rate of 1.167uL/minute with Ringer's Solution (147mM NaCl, 4mM KCl, 1.4mM CaCl2, in distilled water). Probes were primed for at least 30 minutes before baseline collection began at ZT 23. Dialysis samples were collected in fractions of 20 minutes (23.3uL dialysate) for 7 hours, comprising a baseline timeframe of ZT 23-0 and an experimental timeframe of ZT 0-6. Upon collection from the dialysis system, the dialysates were immediately frozen at -20C and stored until HPLC analysis. At all times during collection, animals were allowed to move freely about the cage and were provided food and water *ad libitum*. (See Fig. 41 for timeline)

To assess the effects of E2 on baseline adenosine levels, OVX animals treated with E2 (n=8) or oil (n=11) were allowed to sleep *ad libitum* with the microdialysis probe in place for the first six hours of the light phase. To assess the effects of E2 on adenosine levels during homeostatic sleep need, a second cohort of OVX animals treated with E2 (n=7) or oil (n=8) were subjected to sleep deprivation via gentle handling with the microdialysis probe in place for the first six hours of the light phase.

To calculate an *in vitro* probe recovery rate, free microdialysis probes (n=3) were inserted into a solution of 100nM adenosine (Tocris Biosciences, Bristol, U.K.) in Ringer's Solution, and perfused with Ringer's Solution at 1.167uL/min for 2 hours, with 20 minute dialysate fractions collected.

6. Quantification of Adenosine.

The dialysates were processed by the Proteomics Core Laboratory in the Center for Vascular and Inflammatory Diseases at the University of Maryland, School of Medicine. Adenosine content was quantified by liquid chromatography tandem-mass spectrometry by monitoring the transition pair of m/z 268.1/136.1 and quantified by plotting the area under the curve versus the known concentrations of the standards from a calibration curve. Analysis was performed on a Perkin Elmer QSight LX50 HPLC system and a QSight 210 triple-quadrupole mass spectrometer. The chromatographic solvents used were 0.1% formic acid in water (Solvent A) and 0.1% formic acid in methanol (Solvent B). The column was a YMC Triart 3µ C18, 2.1mm x 150mm operated at a flow rate of 400µl/min at 45°C. An isocratic separation was used with a solvent composition of 94% Solvent A and 6% Solvent B. The effluent from the column was introduced into the mass spectrometer by electrospray ionization in positive polarity and the transition pair of m/z 268.1/136.1 at

unit mass resolution was used for detection of adenosine. The run time was 3min. A stock solution of adenosine (Sigma) was prepared from dry powder and finally diluted in Solvent A to obtain a 6-point calibration curve that ranged from 5pg – 1500pg injected on column. The area under the curve for the adenosine standards was plotted against their known concentrations to quantify the amount of adenosine in the experimental samples.

Experiment 3:

7. Microinfusion of Adenosine A_{2A} Receptor Agonist CGS-21680.

To test whether an interaction between E2 and adenosine signaling in the MnPN impacted sleep behavior, the efficacy of a highly selective A2A receptor agonist, CGS-21680 (CGS) (Tocris Biosciences, Bristol, U.K.) was locally infused into the MnPN. In a within-animal design, sleep behavior was assessed across four conditions comprising a 2X2 matrix of Steroid (E2/oil) and Agonist (CGS/DMSO) conditions. Adult female Sprague-Dawley rats (n=10) were OVX and implanted with EEG/EMG transmitters and a guide cannula targeting the MnPN as described above. A randomly selected cohort was given two doses of sesame oil followed by one infusion of DMSO vehicle. A second randomly selected cohort of animals was given two doses of sesame oil followed by one infusion of CGS. A third randomly selected cohort of animals was given two doses of E2 followed by one infusion of DMSO vehicle. And the final randomly selected cohort of animals was given two doses of E2 followed by one infusion of CGS. These cohorts serve to evaluate estrogenic effects on the ability to stimulate the adenosinergic sleep pressure system. After 7 days of recovery, the animals were subjected to the opposite treatments in steroid treatment and drug interventions for a total of four iterations per animal.

To summarize the experimental manipulations, after one week of recovery from the implantation and ovariectomy surgery described above, the animals were administered E2 or sesame oil vehicle. To avoid direct E2 suppression of sleep behavior, a sub-physiological dose of E2 was administered (1.25µg and 2.5µg). Four hours after the second E2 injection (~ZT 16), the dummy stylets were removed and replaced by 33 gauge microneedles (PlasticsOne) that project to the end of the 6.5mm guide cannulae. Needles were attached via polyethylene tubing to a 25 µl Hamilton syringe (700 series, Hamilton). The flowrate of the syringe was controlled by a BASi Bee pump attached to a Bee Hive controller (Bioanalytical Systems). Infusions of 24nmol (5uL) of CGS-21680 (Tocris Biosciences, Bristol, U.K.) (CGS), or DMSO vehicle (5uL) occurred over 1 min. The dose of CGS was previously reported to markedly increase sleep in male rats when infused into the lateral ventricle,[151] with animals receiving the opposite treatment three days later. (See Fig. 46 for timeline.) This treatment was designed to pharmacologically increase sleep pressure through direct stimulation of the adenosinergic system. Animals were returned to their cages, and sleep behavior was analyzed for eight hours following each injection.

C. Results

1. Estradiol Differentially Affects Sleep in Response to Different Levels of Homeostatic Sleep Need in the Adult Female Rat.

In adult female rats, our previous findings have consistently demonstrated that both endogenous and exogenous E2 suppresses dark phase NREM sleep by ~40%.[80] Based on the principles that govern homeostatic sleep,[156] such findings would predict that E2-mediated sleep reductions induce compensatory increases in homeostatic sleep need, such as increases in sleep duration following the prolonged period of wake and increases in

NREM-SWA. To test, a series of experiments were designed to investigate whether (1) E2 induced increases in dark phase wake result in increased light phase homeostatic sleep need and (2) E2 affects recovery sleep after sleep deprivation. To lessen individual variability seen in sleep-wake behavior, a within-animal design was used to compare the effects of E2 to oil baseline. Treatments were randomized within the cohorts and animals were provided a 7-day washout period of *ad libitum* sleep between experimental manipulations (Fig. 28, 32, and 37). In congruence with previously reported findings,[80] there was no significant difference in sleep-wake durations across the 12- hour light period (Fig. 29).

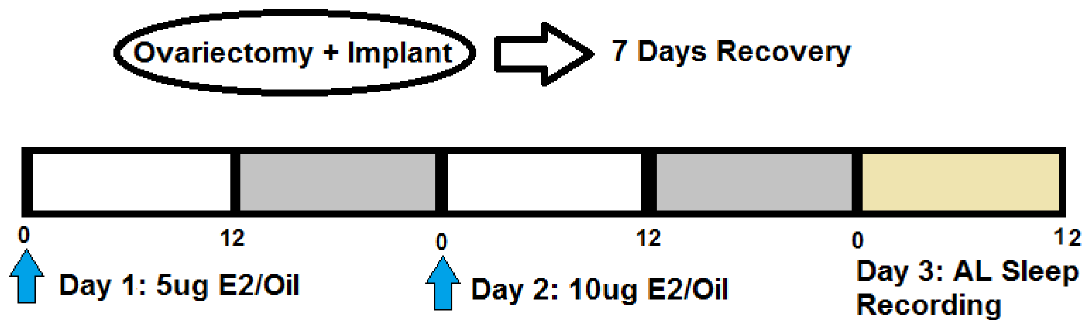


Fig. 28. Timeline of *Ad Libitum* Sleep Experiment. Ovariectomized female Sprague-Dawley rats (n=11) were implanted with transmitters, treated with E2 or oil vehicle, and either subjected to sleep deprivation by gentle handling or allowed to sleep ad libitum for the light phase on Day 3.

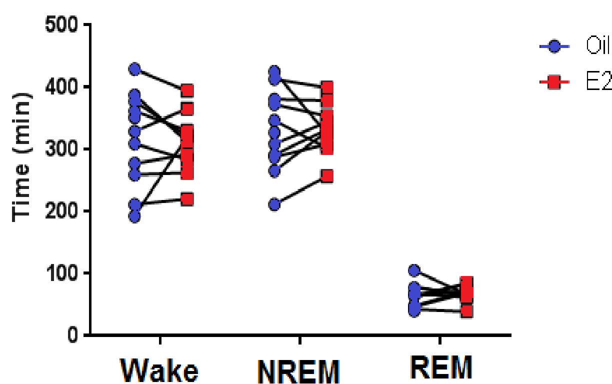
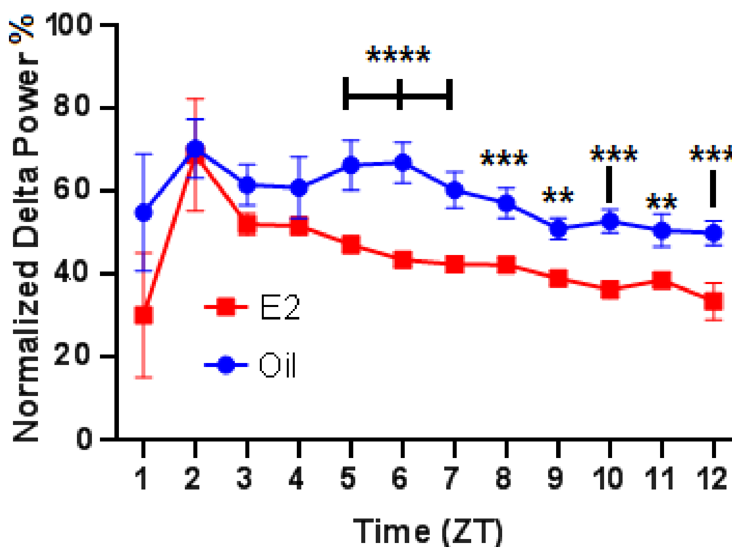


Fig. 29. E2 does not Affect Light Phase Sleep Times. Ovariectomized female Sprague-Dawley rats were implanted with transmitters, treated with E2 or oil vehicle, and allowed to sleep ad libitum for the light phase on Day 3. No significant change in wake time, slow wave sleep time, or paradoxical sleep time was observed, in agreement with prior findings showing lower impact of E2 in the light phase.

a. When Sleeping *ad libitum*, Estradiol-Treated Animals Sleep Equivalent Times with Lower SWA

Analysis of NREM-SWA (0.5-4.5Hz delta band) revealed that E2 significantly decreased the amount of SWA compared to the oil control day (Fig. 30). Spectral analysis of the NREM-SWA frequency distributions in the 0.5-4.0 Hz bands further revealed that E2 treatment decreased the power in the delta band (Fig. 30-31) with significant differences between E2 and oil treatments occurring in the lowest frequency bands including the 0.25Hz, 0.5Hz, 0.75Hz, and 1Hz bins. The analysis of NREM-SWA supports the findings that E2 decreased SWA, despite there being no significant change in sleep-wake behavior. Fourier transformation analysis of spectral components revealed this decrease in power to be concentrated among the lowest frequencies, below 1Hz. The data suggest there is a decrease in the brain EEG waves responsible for the deepest, most homeostatically restorative NREM sleep (Fig. 31), and suggesting E2 reduces homeostatic sleep need in the light phase.

Fig. 30. E2-Treated Animals Show Lower Delta Power When Sleeping *Ad Libitum*. On Day 3, Delta Power during NREM sleep was analyzed in animals sleeping ad libitum for ZT 0-12. E2-treated animals showed significantly lower delta power (p<.0001, two-way ANOVA, main effect of hormone treatment) across the entire light phase. (Repeated Measure two-way ANOVA; main effect of hormone; (F (8, 64) = 9.375, P < 0.0001 overall power) A post hoc multiple comparison revealed significant differences for the majority of hours across the light phase (Sidak's multiple comparison test; p<.0001 ZT5,6,7, p<.001 ZT8,10,12, p<.01 ZT9,11).



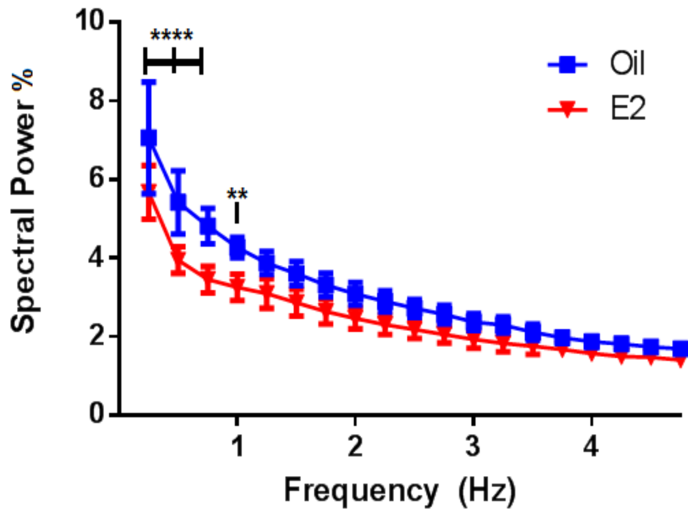


Fig. 31. E2-Treated Animals Show Lower Power in the Low Delta Band. On Day 3, when allowed to sleep *ad libitum* for ZT 0-12, E2-treated animals showed significantly lower power across the frequency spectrum (p=.04, two-way ANOVA, main effect of treatment) with this effect most significant in the lowest frequency ranges (0-1 Hz). (Repeated Measure two-way ANOVA; main effect of hormone; $F_{(1,10)}=5.375$, p=0.04 delta band) (Sidak's multiple comparison test p<.0001 for 0.25-0.75 Hz and p<0.01 for 1Hz)

b. With Deprivation, Estradiol-Treated Animals Sleep Less with Equivalent SWA

Building on the data showing E2 changing NREM-SWA but not sleep time at conditions of physiological sleep pressure, we next attempted to test whether the same effect is observed at conditions of supraphysiological sleep pressure. We tested conditions of high sleep pressure by depriving the animals of sleep for the first six hours of the light phase, when sleep pressure is already high, and using deprivation to drive that sleep pressure to an even higher level. (Fig. 32)

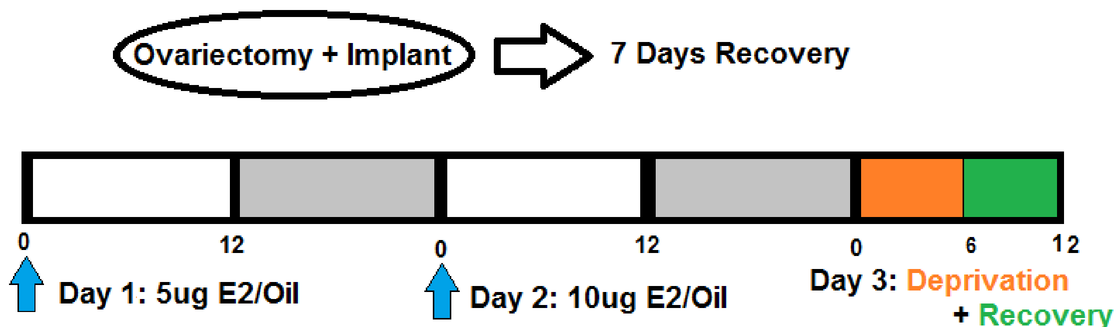


Fig. 32. Timeline of Deprivation Experiment. The same ovariectomized rats in the previous experiment (n=11) were subjected in randomized timing to sleep deprivation by gentle handling on Day 3 for ZT 0-6 and then allowed to recover by sleeping ad libitum for the second half of the light phase, ZT 6-12.

Following 6 hours of sleep deprivation, E2-treated animals show a significant increase in wake time (two-tailed paired t-test $t_{(9)=}$ 3.45, p=0.0073) and decrease in slow wave sleep time (two-tailed paired t-test $t_{(9)=}$ 3.27, p=0.0097) during the deprivation recovery period (Fig. 33). It is important to note that compared to *ad libitum* NREM sleep amounts, the E2 and oil treated females exhibited a sleep rebound with the oil treated females sleeping slightly more than the E2-treated females.

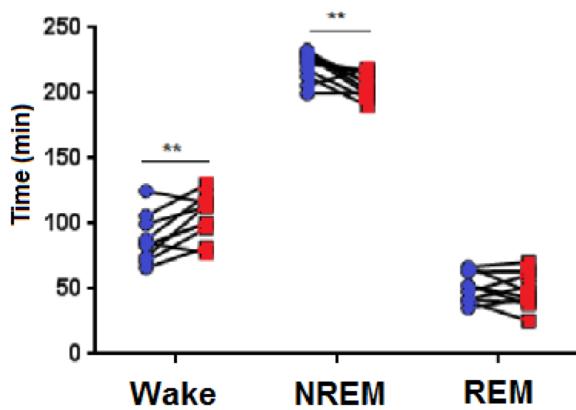


Fig. 33. E2-Treated Animals Show Higher Wake Time and Lower NREM Sleep Time in Deprivation Recovery. E2-treated animals showed a significant increase in wake time and a decrease in NREM sleep time during recovery, but no significant change in REM. (Wake two-tailed paired t-test t(9)= 3.447, p<.01) (NREM two-tailed paired t-test t(9)= 3.269, p<.01)

Recovery NREM-SWA also exhibits the characteristic increase in power indicative of a homeostatic response in both oil and E2 treated females relative to their *ad libitum* baseline (Fig. 34-35). However, the difference in delta EEG power between E2 and oil treated animals seen at baseline is obliterated (Fig. 34) and the E2-treated animals have a correspondingly larger fold change over their lower baseline (Fig. 35). The E2 and oil treated groups show the same delta power across the spectrum in Fourier analysis (Fig. 36). These results suggest that despite equivalent amounts of NREM sleep, E2-treated animals

have less NREM-SWA during *ad libitum* sleep. Conversely, during recovery, E2-treated animals sleep less but the NREM-SWA difference is obliterated.

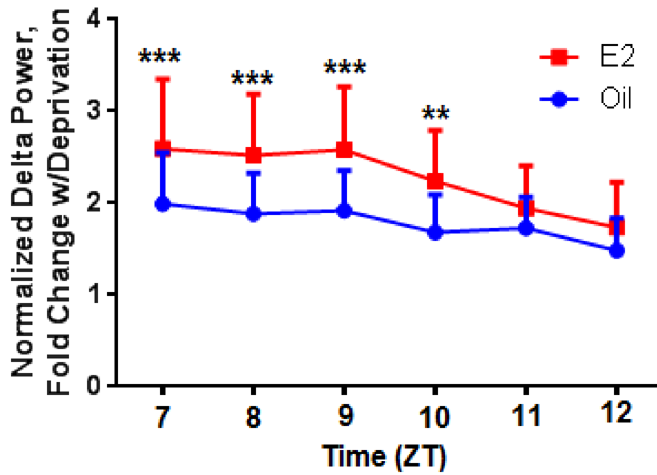


Fig. 34. E2 does not Affect Delta Power in Deprivation Recovery. Delta Power was analyzed in the Day 3 ZT 6-12 recovery phase from sleep deprivation. There was no statistically significant difference in delta power (normalized to ad libitum total power) between E2- and oil treated animals (p=.37, two-way ANOVA, main effect).

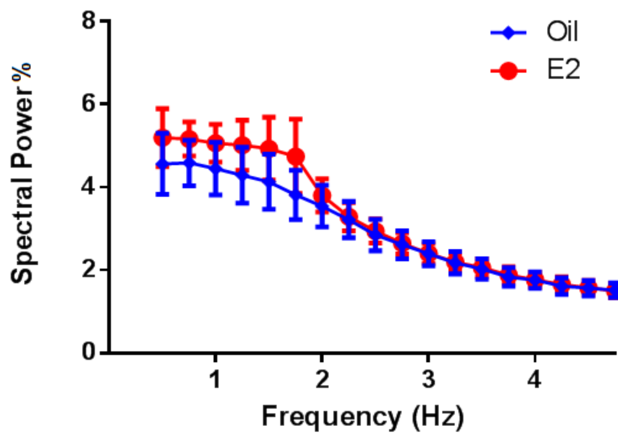


Fig. 35. E2-Treated Animals Show Greater Fold Change in Delta Power from Baseline. Due to the lower baseline values for delta power (Fig. 1B), E2-treated animals exhibited a significantly greater fold increase in NREM Delta Power at ZT 7-9 (p<.001) and ZT 10 (p<.01). (Repeated Measure ANOVA main effect of treatment; $F_{(3,21)}$=7.70, p=.0012; Sidak's multiple comparison test, Ad Libitum Oil vs. Recovery Oil p<.05, Ad Libitum E2 vs. Recovery E2 p<.01) (post-hoc Sidak's Multiple Comparison Test; F (1, 8) = 2.197, ZT 7-8-9 p<.001, ZT 10 p<.01)

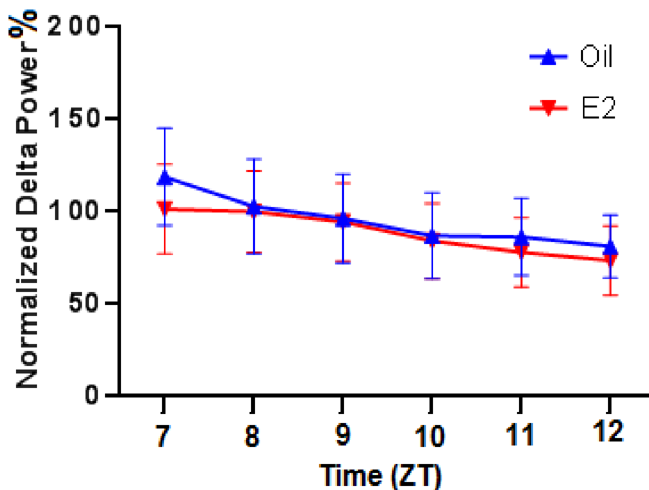


Fig. 36. E2 does not Alter Spectral Power in Deprivation Recovery. There was no statistically significant change in power across the frequency spectrum between E2- and oil treated animals in deprivation recovery (p=.41, two-way ANOVA, main effect).

c. 3-Hour Deprivation Studies Show the Estradiol-mediated Obliteration of SWA Difference is not a Ceiling Effect

To test if the effects were due to the 6-hour deprivation period pushing the animals to a ceiling of achieving the maximum levels of delta power and the most homeostatically restorative sleep possible, overriding any effect of E2, we repeated the experiment with a 3-hour deprivation period in a subset of the animals. (Fig. 37)

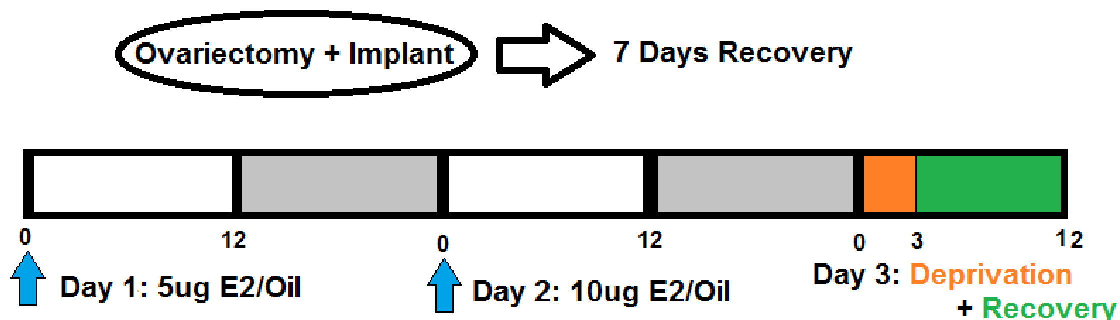


Fig. 37. Timeline of 3-Hour Deprivation Experiments. To test if the effect was due to a ceiling in the ability of the animals to increase NREM Delta Power, a subset of the animals (n=5) were subjected to gentle handling for a 3-hour deprivation period on Day 3 (ZT 0-3) and allowed to recover for the remainder of the light phase (ZT3-12).

With the 3-hour deprivation, the E2-treated animals still showed a significantly higher wake time and lower slow wave sleep during their recovery period relative to oil controls (Fig. 38). The 3-hour deprivation was also sufficient to obliterate the difference between E2 and oil treated groups in delta power during recovery (Fig. 39) as seen in the 6-hour deprivation. Together, analysis of NREM-SWA suggests that E2 did not greatly dampen the compensatory response to a prolonged sleep deprivation, however, E2 did attenuate baseline homeostatic responses by decreasing NREM Delta Power. Thus, E2 may be working in the MnPN to increase the homeostatic set-point for sleep, allowing for a decrease in sleep propensity despite periods of increased wake duration.

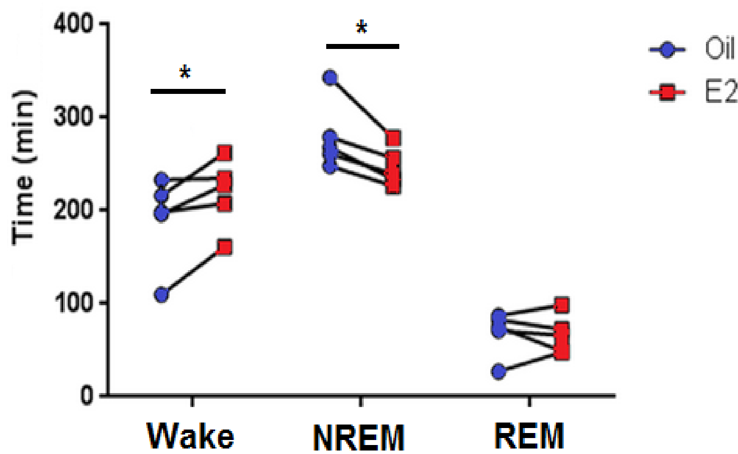


Fig. 38. E2 Increases Wake and Decreases NREM Sleep in 3-Hour Deprivation Recovery. A similar increase in wake time (p<.05) and decrease in sleep time (p<.05) in E2-treated animals relative to oil controls was observed as in the 6-hour deprivation. (Wake two-tailed paired t-test $t_{(4)}=$ 2.832, p<.05) (NREM two-tailed paired t-test $t_{(4)}=$ 3.967, p<.05)

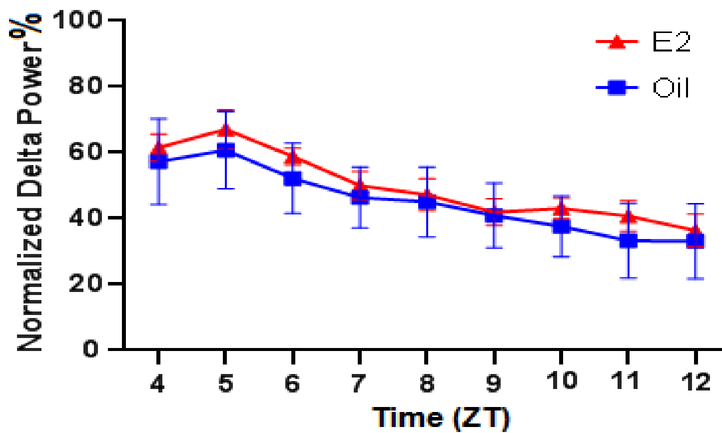


Fig. 39. E2 does not Affect Delta Power in 3-Hour Deprivation Recovery. Animals subjected to the 3-hour deprivation did not show any significant difference in delta power in E2-treated animals versus oil controls.

d. Estradiol Increases Wake Bout Length and Decreases Slow Wave Sleep Bout Length in Deprivation Recovery

To test whether the sleep behavior changes are due to fragmentation of sleep, an effect which has been shown with E2 in previous studies,[80,204-205] further analysis of the sleep architecture was performed by plotting bout length on a cumulative probability distribution. Relative to oil-treated controls, E2-treated animals showed neither longer or shorter overall wake bouts when sleeping ad libitum, as well as no significant difference in NREM or REM sleep bout distribution. (Fig. 40A-C-E). However, in deprivation recovery, E2-treated animals showed longer wake bouts (Fig. 40B), shorter slow wave sleep bouts (Fig. 40D), and shorter REM sleep bouts (Fig. 40F) relative to oil controls. These results show that the changes in sleep times seen with E2 in deprivation recovery are not due to a

fragmentation of sleep and wake, but rather a lengthening of consolidated wake and a shortening of consolidated sleep times. These results suggest that E2 affects the homeostatic sleep behavior of the animals, pushing behavior into the wake state at times when sleep would be attained without E2.

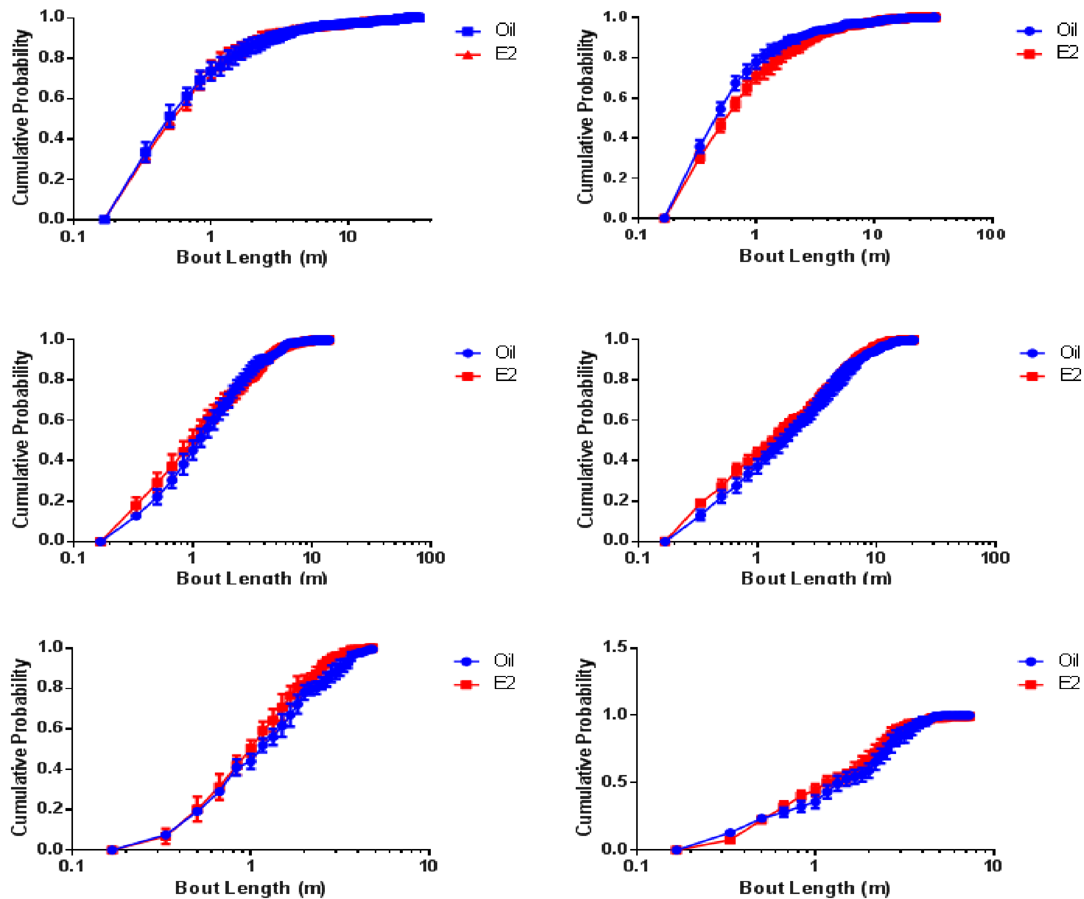


Fig. 40 A-B-C-D-E-F. E2 Increases Wake Bout Length and Decreases Slow Wave Sleep Bout Length in Deprivation Recovery. To determine if sleep architecture differed between E2-treated animals and oil controls, sleep bout length was analyzed in a cumulative probability distribution for wake, NREM, and REM sleep stages. **(A)** Relative to oil controls, E2-treated animals showed a different distribution of wake bouts at baseline ($p<.0001$) without a shift toward longer or shorter bouts. **(B)** E2-treated animals show longer wake bouts in deprivation recovery. ($p<.0001$) **(C)** There was no significant change in slow-wave sleep bout length at baseline. ($p=.27$) **(D)** E2-treated animals show shorter slow wave sleep bouts in deprivation recovery ($p=.02$) **(E)** There was no significant change in REM sleep bout length at baseline ($p=.56$) **(F)** E2-treated animals show shorter REM sleep bouts in deprivation recovery ($p=.003$) (p-values Wilcoxon Signed-Rank test)

2. Estradiol Treatment Increases Adenosine Levels in the MnPN in Adult Female Rats

Building on the finding that E2 changes levels of homeostatic sleep pressure by behavioral metrics, we next attempted to determine if E2 changes molecular markers of homeostatic sleep need, both under physiological *(ad libitum)* and supraphysiological (deprivation) conditions. To do so, we measured adenosine, intracranial concentration of which has been shown to correlate with homeostatic sleep need.[149] Building on previous findings[159] showing the Median Preoptic Nucleus is a site of hormonal effects on sleep, we attempted to determine if E2 is affecting adenosine levels in the MnPN under conditions of both physiological and supraphysiological sleep pressure.

a. Estradiol Increases MnPN Adenosine Levels Relative to Oil when Sleeping *ad libitum*

To test whether E2 induced reductions in NREM-SWA are associated with reduced levels of extracellular adenosine in the MnPN, we analyzed microdialysates from OVX females treated with Oil or E2 according to our standard treatment paradigm. We employed the technique of microdialysis and HPLC-Mass Spectrometry, which has been used in prior studies[206-207] to measure extracellular adenosine content in the brain. (Fig. 41) For those animals that were histologically determined to be preoptic area cannulation hits (Fig. 42), adenosine levels were measured. Samples collection began 24 hours after the last E2 injection at ZT0 and continued through the light phase. We chose to collect in the light phase to maximize the probability of (1) detecting adenosine when levels should be highest (i.e. at the start of the light phase) and (2) measuring differences in the accumulation of adenosine after a period of dark phase activity. To control for differing baseline adenosine levels between animals, adenosine content was normalized to each animal's baseline of ZT

23-0, just before the onset of the light phase and any deprivation intervention; data are reported as a percent of the dark phase baseline. Using *in vitro* recovery to calculate the probe recovery rate of 12%, we determined that the average baseline adenosine concentration in the CSF of the animals is 203nM, similar to literature reference ranges of 50-200nM.[208]

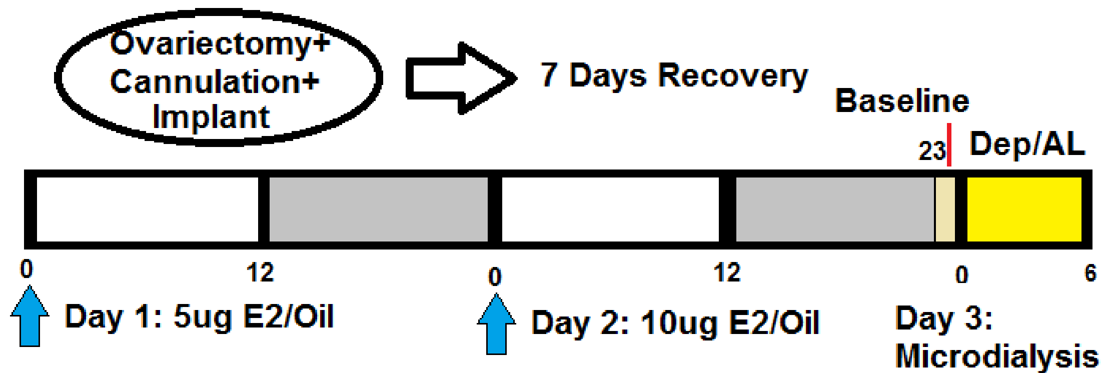


Fig. 41. Timeline of Microdialysis Experiments. Ovariectomized female Sprague-Dawley rats were implanted with microdialysis guide cannula to the MnPN and transmitters, treated with E2 (n=8) or oil vehicle (n=11), and allowed to sleep ad libitum for the first half of the light phase (ZT 0-6). Adenosine levels were collected by microdialysis (6kd-cutoff probes) and analyzed by HPLC-Mass Spectrometry. Adenosine levels were normalized to each animal's baseline adenosine collected from ZT 23-ZT 0.

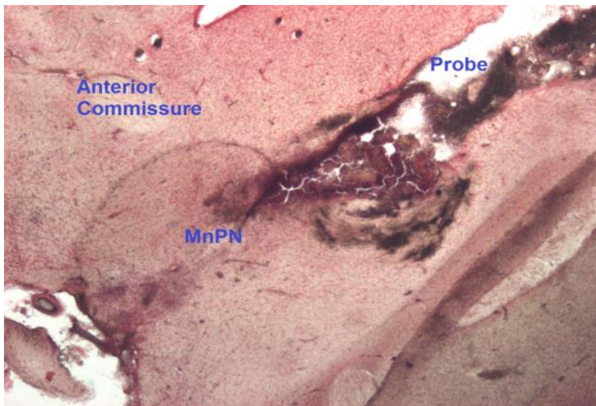
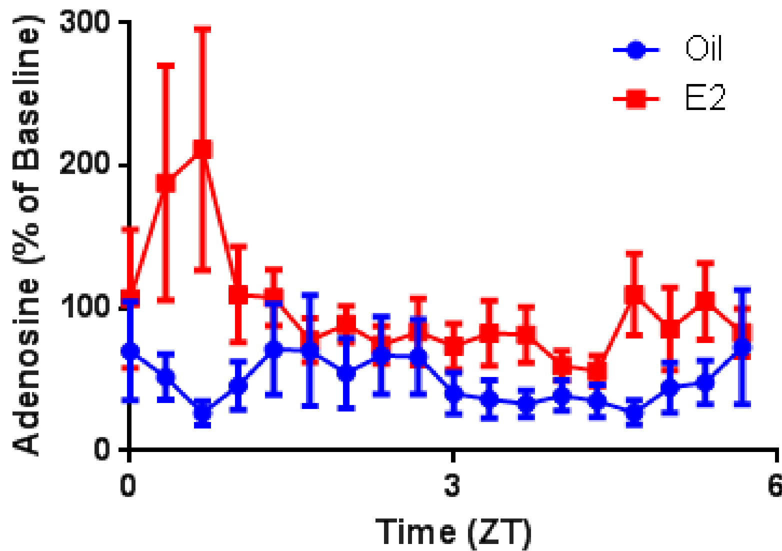


Fig. 42. Microdialysis Probes are Targeted to the Preoptic Area. We have developed coordinates targeting the preoptic area; a representative probe hit (right) is shown. Probe placement was determined visually using neutral red staining and misses were excluded.

Without deprivation, adenosine levels in both groups decrease, as expected due to the animals being allowed to sleep and in line with prior experiments of microdialysis from the preoptic area in cats.[206] However, across the light phase, adenosine content was significantly greater in E2-treated females compared to the oil controls (Fig.

43). In particular, the oil control level of adenosine decreases faster and to a lower final adenosine level than E2-treated animals, while conversely the decrease in the E2-treated animals is significantly blunted relative to oil controls. Moreover, E2 significantly increased total adenosine by over 2-fold. This increase took place despite the finding, from animals implanted with transmitters before microdialysis, that E2- and oil-treated animals collected the same amount of sleep despite their differing adenosine levels (Fig. 44).

Fig. 43. E2-Treated Animals Have Higher Preoptic Adenosine Levels in *Ad Libitum* Sleep. E2-treated animals showed a consistently higher level of adenosine when sleeping ad libitum throughout the ZT0-6 period (Repeated Measure ANOVA, main effect of treatment and interaction between treatment and time; $F(1, 14)=13.19$; $p<0.005$ and $F(18, 246)=2.580$; $p<0.001$, respectively). AUC significantly increased (AUC, Welch-corrected two-tailed t-test, $t(12.52)=3.973, p<0.002$).



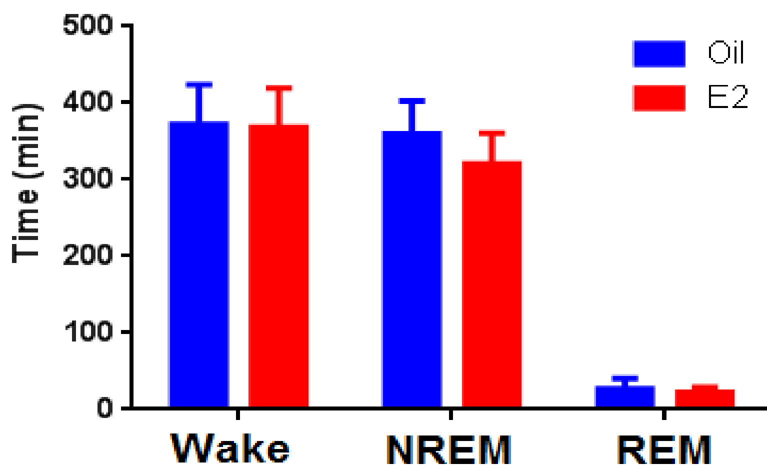


Fig. 44. Microdialysis does not Affect Sleep Times. A subset of animals (n=4 E2, n=4 oil) were implanted with transmitters before microdialysis. In these animals we did not see changes in ad libitum sleep time in the light phase with E2, in line with results from animals not undergoing microdialysis.

b. Estradiol Also Increases MnPN Adenosine Levels Relative to Oil with Deprivation

When the animals were subjected to deprivation, similar results held at higher overall levels of adenosine. E2-treated animals showed a significantly higher level of adenosine throughout the deprivation time period, rising from baseline, while oil-treated animals remained largely flat in their adenosine levels. (Fig. 45) These results suggest that E2 is not acting to reduce homeostatic sleep pressure as marked by total adenosine content in the preoptic area.

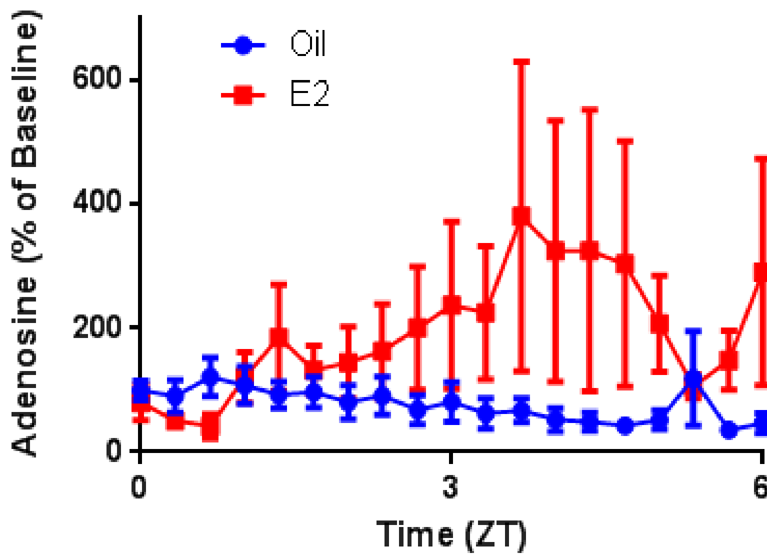


Fig. 45. E2 Increases Preoptic Adenosine Levels with Deprivation. Ovariectomized female Sprague-Dawley rats were implanted with microdialysis MnPN guide cannula and transmitters, treated with E2 (n=7) or oil vehicle (n=8), and sleep-deprived by gentle handling for the first half of the light phase (ZT 0-6). Adenosine levels were collected by microdialysis (6kd-cutoff probes by SciPro, Sanborn, N.Y.) and analyzed by HPLC-Mass Spectrometry. Adenosine levels were normalized to each animal's baseline adenosine level collected from ZT 23-ZT 0. E2-treated animals showed a consistently higher level of adenosine when sleeping ad libitum throughout the ZT0-6 period (Repeated Measure ANOVA, interaction between hormone treatment and time; $F_{(18, 162)}=2.172$, $p<.01$.)

3. Estradiol Treatment Impairs the Ability to Pharmacologically Stimulate Sleep Behavior via the Adenosinergic System in Adult Female Rats

As adenosine has been shown to be both a molecular marker and mediator of sleep pressure,[149-150] we next attempted to test whether the higher levels of adenosine in the MnPN are changing the ability of adenosine to act as a somnogen mediator of homeostatic

sleep pressure. To test that prediction, we pharmacologically stimulated the adenosinergic sleep pressure system, both with and without E2. We locally pharmacologically stimulated the adenosinergic sleep pressure system using the A2A receptor agonist CGS21680, which activates pro-sleep adenosine receptors in the MnPN. CGS or DMSO infusions were conducted early in the dark phase, at ZT16, to capitalize on the increasing homeostatic sleep need of the animals, and thus attempt to flip the homeostatic sleep behavior switch.[84] In a randomized within-animal design, animals received 1) Oil+DMSO Vehicle, 2) Oil+CGS-21680, 3) E2+DMSO Vehicle, and 4) E2+CGS-21680, with 7-day washout periods between each treatment.

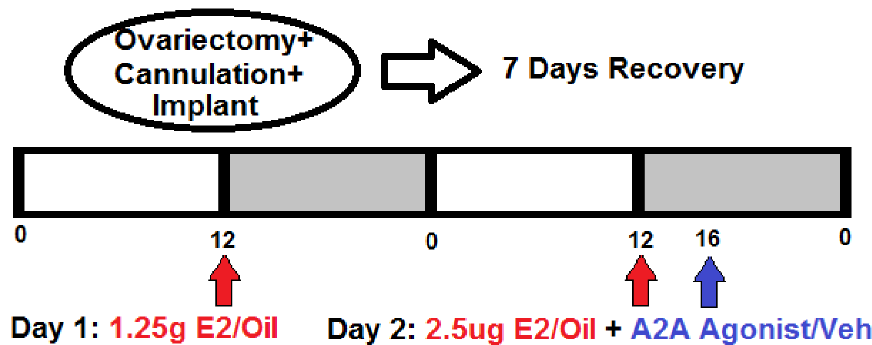


Fig. 46. Timeline of A2A Agonist Treatment Experiment. Ovariectomized Sprague-Dawley rats (n=10) were implanted with transmitters and guide cannula to the MnPN. After recovery, E2 or oil vehicle hormone replacement was conducted at a dose ¼ of our standard paradigm. On the day of highest E2 effects on sleep (Day 3), 24nmol of the A2A agonist CGS-21680 in 5uL DMSO, or 5uL DMSO was infused into the MnPN at ZT 16 and sleep times were quantitated for the remainder of the dark phase.

a. Levels of Estradiol that do not Affect Sleep *per se* Impair the Effect of Adenosine 2A Agonist CGS21680

The low-dose E2 treatment did not produce effects on wake or sleep times per se (Fig. 47-48) but did decrease REM sleep without the agonist on board (Fig. 49), in concert with results from prior studies.[80] In oil treated animals, the infusion of agonist led to a decrease in wake time and increase in slow wave sleep time as expected, a result equivalent

to that in male animals.[151] However, when agonist treatment was conducted in the E2-treated animals, the ability of the adenosine agonist to effect an increase in slow wave sleep time and decrease in wake time was significantly attenuated. (Fig. 50-51). The agonist did not have a significant effect on REM sleep with or without E2 (Fig. 52). These results show that E2 is acting on the adenosinergic system to decouple adenosine somnogen content in the preoptic area from sleep behavior.

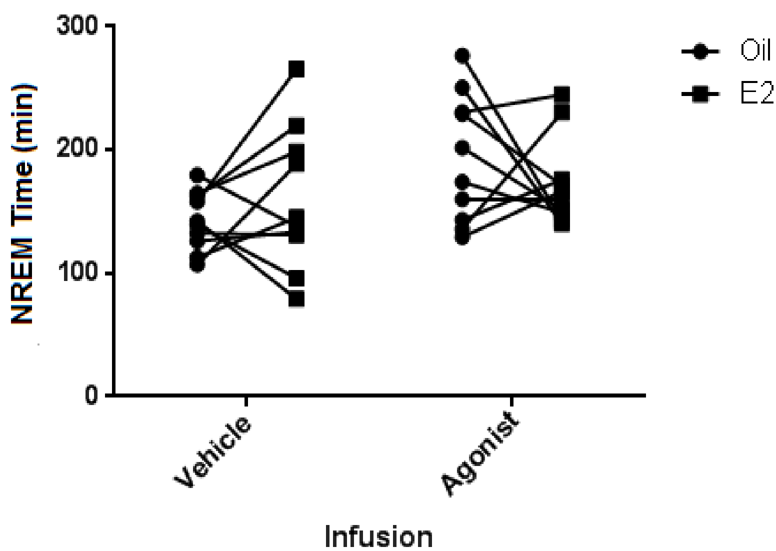


Fig. 47. Low-Dose E2 does not Affect Wake Time. This hormonal dose was not enough to affect wake time in the last 8 hours of the dark phase.

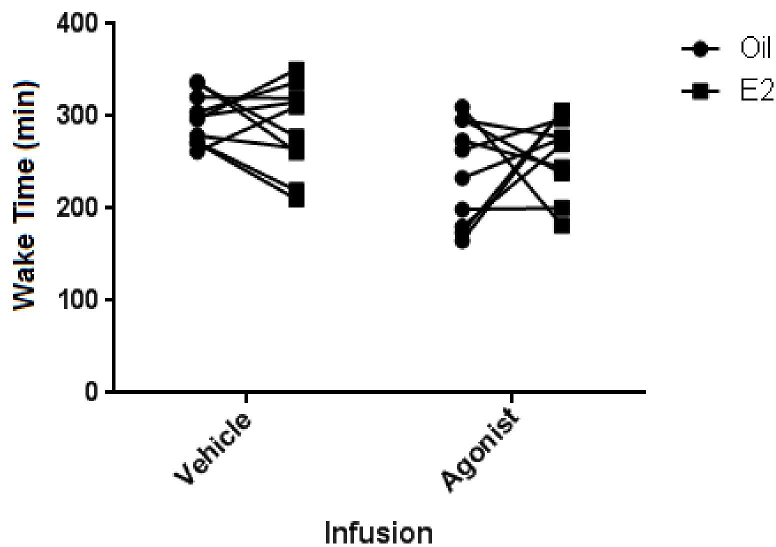


Fig. 48. Low-Dose E2 does not Affect NREM Sleep Time. This hormonal dose was not enough to affect NREM time in the last 8 hours of the dark phase.

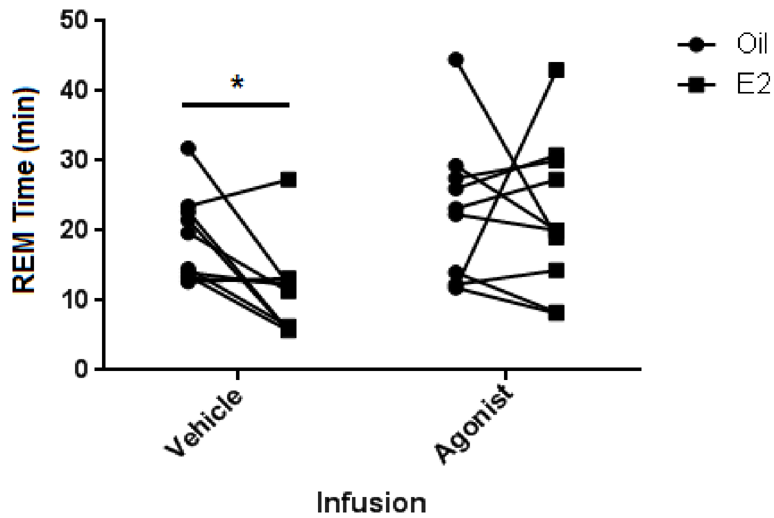


Fig. 49. Low-Dose E2 Reduces REM Sleep Without the Presence of A2A Agonist. The low dose of E2 did significantly reduce REM Sleep time (p=.01, two-way ANOVA, main effect of hormone) when no adenosine agonist was infused, in line with previous studies showing REM sleep acutely responsive to small changes in hormonal fluctuations.

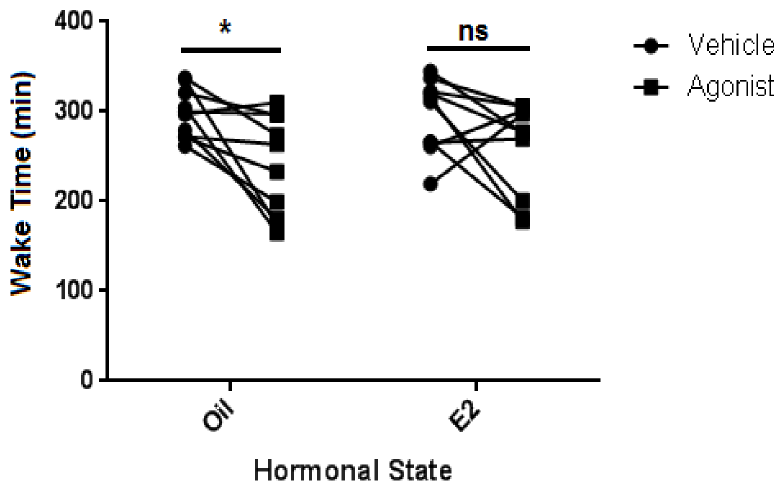


Fig. 50. In Oil-Treated Animals Only, A2A Agonist Increases Wake Time. Similar results in male animals, the agonist significantly decreased wake time in oil-treated animals. However, when animals were treated with E2, there was no significant change in wake times. (Repeated Measure ANOVA with post-hoc Sidak's Multiple Comparison Test; $F_{(1,9)}$=2.581, Vehicle vs. Agonist in Oil p<.05)

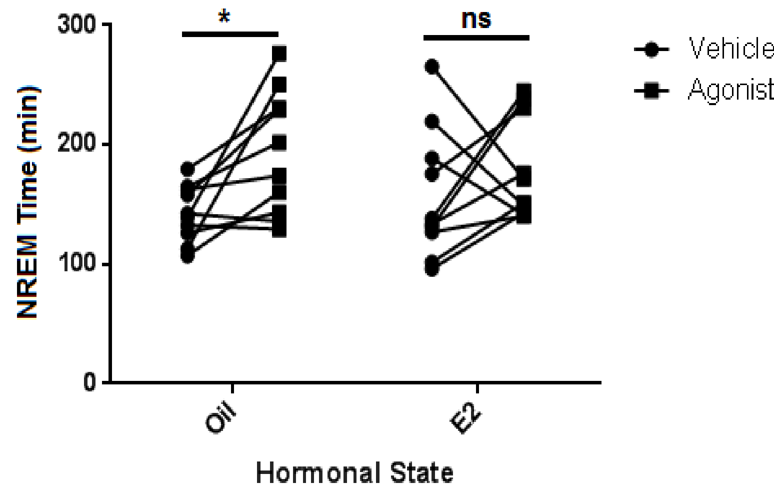
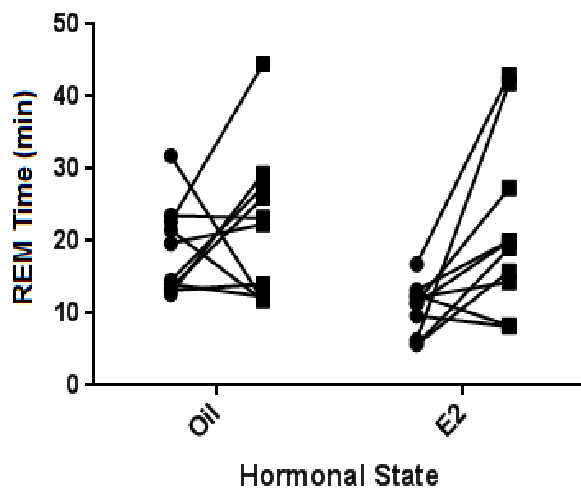


Fig. 51. In Oil-Treated Animals Only, A2A Agonist Decreases NREM Sleep Time. Similar results in male animals, the agonist significantly increased NREM sleep time in oil-treated animals. However, when animals were treated with E2, there was no



significant change in NREM sleep time. (Repeated Measure ANOVA with post-hoc Sidak's Multiple Comparison Test; $F_{(1,9)}=1.818$, Vehicle vs. Agonist in Oil $p<.05$)

Fig. 52. A2A Agonist does not Affect REM Sleep Times. The agonist did not have a significant effect on REM sleep with or without E2 treatment.

b. Both Estradiol and CGS-21680 Decrease NREM Slow Wave Activity

To further investigate these results, we analyzed the EEG power spectra through Fourier analysis for the dark phase period immediately following CGS injection (ZT 16-0). Analysis of the power frequency distribution of NREM-SWA in the oil/vehicle and E2/vehicle groups supported the findings that E2 decreases dark phase SWA (Fig. 53) despite there being no significant change in sleep-wake behavior, when CGS is not present, as well as decreasing power across the frequency spectrum. (Fig. 53A-B). Interestingly, in oil treated females, local infusion of CGS into the MnPN also resulted in a significant lowering of NREM-SWA (Fig. 53A-B). This may represent the decreased sleep need resulting from the increase in the NREM behavior, showing that stimulating sleep behavior exogenously, without having generated the requisite homeostatic need, reduces the level of delta power and the efficiency of that sleep at resolving homeostatic need. Finally, the E2/CGS group also exhibited a decrease in NREM-SWA compared to the oil/veh controls that was not different from the E2/veh or oil/CGS groups. (Fig. 53A-B) Taken together,

these findings further support the possibility that in the MnPN E2 may increase the homeostatic set-point for sleep allowing for a decrease in sleep propensity.

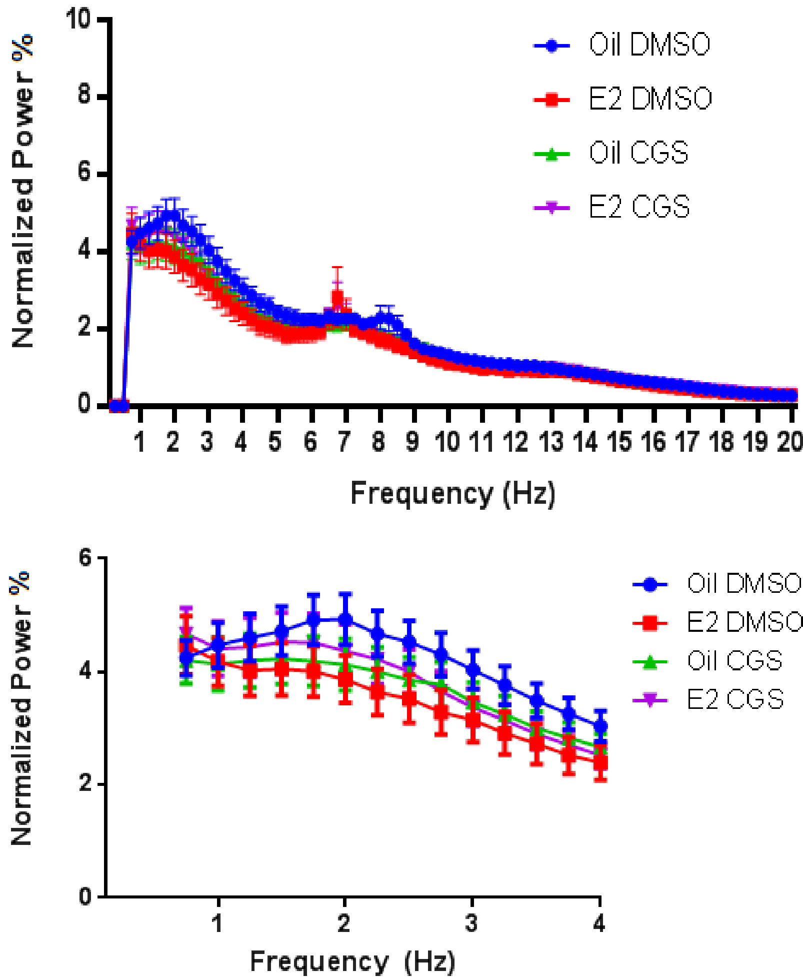


Fig. 53 A-B. A2A Agonist and E2 Both Decrease Spectral Power in the Delta Band in Oil-Treated Animals. **(A)** Analysis of EEG power spectra was performed during the time period ZT 16-0. E2+vehicle treated animals with vehicle showed a significant decrease relative to their oil+vehicle treated counterparts, (Repeated Measure ANOVA, main effect of treatment; $F_{(3, 486)}=2.651$, $p=0.0482$.) in concert with previous work showing E2 is sufficient to lower EEG power in the delta range. The oil+CGS treated animals also showed a significantly lower power over the range of 1-4Hz relative to their oil+vehicle counterparts; Repeated Measure ANOVA, main effect of drug; $F_{(77,702)}=1.987$, $p<0.0001$; interaction of drug and frequency $F_{(1,702)}=97.97$, $p<0.0001$). the E2/CGS group also exhibited a decrease in NREM-SWA compared to the Oil/Veh controls that was not different from the E2/Veh or Oil/CGS groups. The same results at the same significance were present over **(B)** the delta power range of 0.5-4Hz that is important for homeostatically restorative sleep.

D. Discussion

Understanding the role played by sex hormones in sleep regulation offers a deeper insight into why women are at a significantly higher risk for insomnia and sleep disruption. Work in both rats and mice demonstrates that in the absence of circulating sex steroids, sex

differences in sleep behavior and architecture are eliminated,[76-77,79-80] suggesting that sex differences in sleep are primarily dependent on circulating sex steroids. Our previous work in adult female rats demonstrates that sleep-wake behavior and activation of preoptic area sleep nuclei are highly sensitive to fluctuations in circulating E2.[7,80] In the present study, we have extended our investigation of the relationship between E2 and homeostatic sleep pressure. Our findings have demonstrated that E2 has different effects on sleep time and NREM-SWA, a measure of homeostatic efficiency of sleep,[109] under conditions of normal sleep and sleep deprivation. E2 serves to decrease NREM-SWA but not sleep time, in the light phase under normal sleep, while in deprivation recovery, E2 decreases sleep time but not NREM-SWA. These differences suggest that E2 has effects that interact with the homeostatic sleep pressure system. We further found that E2 increased levels of extracellular adenosine, a measure of homeostatic sleep need,[149] under both normal and deprivation sleep conditions. To resolve the discrepancy between the decrease in behavioral markers associated with homeostatic sleep need and increase in levels of adenosine, we employed an agonist of the A2A receptor to stimulate the adenosinergic sleep pressure system. These studies show that E2 blunts the ability of the A2A agonist to drive sleep behavior, showing an interposition of E2 into the sleep pressure system that blunts the ability of adenosine to act as a somnogen. Overall, these experiments show an interaction between E2 and adenosinergic homeostatic sleep pressure.

Sleep pressure has been described as the intrinsic need for sleep of a given animal at a given time.[112] Two sleep-pressure systems, known as the circadian wake system and the homeostatic sleep pressure system, operate in concert to generate an overall sleep pressure that is responsive to both the animal's intrinsic homeostatic needs as well as

external factors such as the light-dark cycle.[5] As the name suggests, the homeostatic sleep system governs the amount of sleep needed after a given period of wake to maintain homeostasis.[124] The total amount of sleep needed for an animal in a given period of time tends to be quite consistent, and independent of both the circadian system and the light-dark cycle.[124] This phenomenon of homeostatic sleep is further exemplified by the need for recovery sleep, which is nearly always necessary after periods of sleep deprivation.[125] Homeostatic sleep pressure increases roughly linearly with increasing wake time, reaching a maximum at the onset of the sleep state, and then decreases roughly linearly with time spent asleep.[124] Previously published data suggest that E2 is reducing the sleep obtained by animals in the dark phase,[7,80] implying that the animals should then exhibit a homeostatic rebound in the light phase after this physiological form of deprivation. Our experiment was therefore designed to test both conditions of physiological sleep pressure (*ad libitum* sleep) and supraphysiological sleep pressure (exogenous sleep deprivation). Our data have shown that E2-treated animals require a lower amount of recovery sleep time following deprivation, suggesting that E2 is either lowering the intrinsic need for sleep or that E2 is changing the sleep mechanisms to alter the recovery sleep response to deprivation.

Quantitative markers derived from EEG outputs exist that can approximate sleep pressure in a reproducible fashion.[5] Sleep intensity, as measured by EEG delta power (0.5-4.5Hz) during NREM sleep (NREM-SWA), is a characteristic hallmark of deep sleep need.[129] SWA is generated by synchronized cortical activity during NREM sleep. Increases in the intensity or amount of NREM-SWA is proportional to the amount of prior wake time, and time spent in NREM sleep will dissipate the concentration of SWA. Given these dynamic changes proportional to sleep loss/gain, NREM-SWA is widely used as a reliable

marker of sleep homeostasis.[109,129] Together, our analysis of NREM-SWA suggests that E2 did not greatly dampen the compensatory response to a prolonged sleep deprivation and allowed for deep, homeostatically restorative sleep under those conditions. However, E2 did attenuate baseline homeostatic responses by decreasing NREM-SWA in the light under *ad libitum* sleep conditions. Thus, our data suggest that E2 may be working to increase the homeostatic set-point for sleep, allowing for a decrease in sleep propensity despite increased wake duration.

Previous studies have shown that steroid-induced changes in sleep in female rats are driven by E2. However, what has remained largely undetermined is how and where E2 is influencing the sleep circuitry. Over the past decade, numerous studies using various techniques have demonstrated that neurons in the Median Preoptic Nucleus (MnPN) are involved in sleep-regulatory mechanisms.[159-160] The MnPN (i) has a predominant number of sleep-active cells (i.e., the number of Fos-ir neurons increases following episodes of sustained sleep but not sustained waking),[84,160] (ii) has a high concentration of neurons with elevated discharge rates during both NREM and REM sleep compared to waking (i.e. sleep-active discharge pattern),[172] and (iii) is thought to function to promote and sustain sleep by inhibiting key arousal centers via descending GABAergic (MnPN) and GABAergic/galaninergic (VLPO) projections.[166,169-171] Furthermore, blocking E2 action directly in the median preoptic nucleus of female rats attenuates E2 suppression of sleep.[7] Thus, the MnPN, which has been shown to be E2-sensitive, is an opportune site of investigation for E2 effects on sleep regulation.

An entire class of molecules known as somnogens have been identified that appear to increase homeostatic sleep pressure, including the nucleoside adenosine, produced in the

brain both purposefully and as a waste product of metabolism. Adenosine has been shown to accumulate in the brain, particularly in the basal forebrain, with increasing wake time and decrease in the sleep state.[149,207] While several other molecules have been hypothesized to act in similar fashions to stimulate the sleep pressure homeostat, adenosine is particularly interesting for its dual role as both a marker and a mediator of sleep pressure. If E2 is altering the homeostatic set-point for sleep, one prediction would be that extracellular adenosine would be decreased in the presence of E2. In animals allowed to sleep *ad libitum* in the light phase, extracellular adenosine decreased in both E2- and oil-treated animals, in line with previously published studies of adenosine levels in the preoptic area of cats.[208] However, the decrease in the oil-treated animals was steeper than in E2-treated animals. Under deprivation conditions, E2-treated animals increased adenosine levels, while levels were largely flat in oil-treated animals.

The finding that E2 increased MnPN adenosine content while reducing NREM-SWA presents an interesting paradox which suggests that E2 may be working to dampen the detection of homeostatic sleep need (i.e. adenosinergic signal) and suggests a connection between the effects of E2 and adenosine. It is widely accepted that adenosine's sleep-inducing actions are mediated through the A1R and A2AR present in key sleep and arousal nuclei.[155] Key pharmacological experiments demonstrate that infusion of a highly selective A2AR agonist, CGS 21680 (CGS) into the lateral ventricle of male rats profoundly increases NREM and REM sleep.[151] Other work focusing on the preoptic area sleep active nuclei suggest that activation of A2AR with CGS activates sleep-active neurons in the VLPO[209] and MnPN[151] and increases GABA release in key arousal centers like the TMN.[210-211] To our knowledge, these studies were only done in male rodents and

no prior study has directly infused a A2AR agonist into the MnPN, nor treated females with A2AR agonists against differing hormonal backgrounds. Furthermore, a direct link has been shown between estrogens and A2AR, as estrogens have been shown to decrease expression of wake-inhibiting adenosine 2A receptors.[89]

To attempt to resolve the discrepancy between lower sleep time and NREM-SWA and higher preoptic adenosine in E2-treated animals, we designed an experiment to activate the A2A receptor and therefore stimulate the adenosinergic system. Our results show that injection of an A2A agonist is sufficient to stimulate sleep in females without E2 treatment, as it is in males. However, when E2 is present in those same animals, even at a low dose that does not affect sleep *per se*, the ability of the agonist to stimulate sleep, and thus the ability of activation of the adenosinergic sleep-pressure system to stimulate sleep behavior, is impaired. These results, combined with the sleep behavior and adenosine level data, suggest that E2 is working to disconnect the buildup of adenosinergic sleep pressure from the ability to flip the sleep switch to drive sleep behavior, particularly deep and homeostatically restorative slow-wave NREM sleep.

The molecular mechanisms of hormonal impact on sleep are poorly understood, and studies investigating where and how female steroids act on the brain are only an emerging area of investigation. Steroid receptors, particularly for estrogen, are present throughout the brain and prevalent on multiple sleep-regulating nuclei such as the basal forebrain.[149] Downstream, the orexinergic wake-promoting system of the lateral hypothalamus receives inputs from the MnPN and is highly sensitive to fluctuations in endogenous and exogenous ovarian steroids,[5] suggesting that this section of the homeostatic sleep/wake circuitry may be a key site for estrogen action. The multiple

potential sites of E2 action present an interesting question of how E2 is working to disconnect molecular measures of sleep need from sleep behavior thought to be characteristically responsive to that need. We acknowledge that these experiments are heavily focused on the MnPN as one potential key site and that more global actions of E2 within other sleep and arousal circuits may also be important. However, given that we understand so little about how E2 is influencing sleep, expanding these studies to other brain regions and molecular mechanisms in the future could provide more insight into how E2 and adenosine interact to affect sleep.

IV. Discussion and Conclusions

A. Discussion

Previous research studies using rodent models describe the changes in sleep across the female estrus cycle and following gonadectomy.[7-8,76,80] Studies consistently reported that E2 suppresses NREM sleep and REM sleep in females, while changes in gonadal steroids cause little to no change in sleep in males. Here, we sought to address the mechanism by which proestrus levels of cycling ovarian steroids suppress sleep in females. We show that after hormone replacement of proestrus levels of E2, the suppression of sleep by endogenous hormones may be recapitulated. We further show that this suppression is due to the high levels of E2 alone, and that progesterone, the other major circulating ovarian steroid, did not have a significant impact on sleep behavior. Extending these findings, we found that E2 has direct actions within the sleep-active POA, specifically in the MnPN, which contains estrogen receptors (ERs). Antagonism of ERs in the MnPN, but not the VLPO, attenuated the E2-mediated suppression of both NREM and REM sleep. Finally, we found that, in addition to E2 actions at the MnPN being necessary for sleep suppression, it is also sufficient, as the direct infusion of E2 into the MnPN suppressed sleep with no other intervention. Based on our findings, we predict that proestrus levels of E2 alone, acting at the MnPN, mediate sex-hormone driven suppression of sleep in female rats.

Furthermore, our findings have demonstrated that E2 has different effects on sleep time and NREM-SWA, a measure of the homeostatic efficiency of sleep, under conditions of normal sleep and sleep deprivation. E2 serves to decrease NREM-SWA, but not sleep time, in the light phase under normal sleep, while in the recovery phase after sleep deprivation, E2 decreases sleep time but not NREM-SWA. This difference suggests that

E2 has effects that interact with the homeostatic sleep pressure system. We further found that E2 increased levels of extracellular adenosine, a measure of homeostatic sleep need, under both normal and deprivation sleep conditions relative to oil controls. To resolve the discrepancy between the decrease in behavioral markers associated with homeostatic sleep need and increase in levels of adenosine, we employed an agonist of the A2A receptor to stimulate the adenosinergic sleep pressure system. These studies show that E2 blunts the ability of the A2A agonist to drive sleep behavior, showing an interposition of E2 into the sleep pressure system that attenuates the ability of adenosine to act as a somnogen. Overall, these experiments show an interaction between E2 and adenosinergic homeostatic sleep pressure at the level of the MnPN.

1. Estradiol Acts at the MnPN to Affect Sleep Times

The molecular mechanisms and site of action of hormonal impacts on sleep are poorly understood, and studies investigating where and how female steroids act on the brain are only an emerging area of investigation. From our findings, we further predict that E2 is both necessary and sufficient to reduce the activation of MnPN sleep active cells, and release the MnPN-driven inhibitory tone on downstream targets; however, the pathway of E2 action on sleep is likely far broader. Steroid receptors, particularly for estrogen, are present throughout the brain and prevalent on multiple sleep-regulating nuclei such as the basal forebrain.[149] Downstream, the orexinergic wake-promoting system of the lateral hypothalamus receives inputs from the MnPN and is highly sensitive to fluctuations in endogenous and exogenous ovarian steroids,[5] suggesting that this section of the homeostatic sleep/wake circuitry may be a key site for estrogen action.

Since ICI had little to no effect within the VLPO, while E2 in the MnPN was sufficient to induce changes, E2 is most likely acting predominantly on the MnPN and *not* acting directly on the neural circuits of the VLPO; however, indirect actions on the VLPO via MnPN neurons seem likely to be present. The decrease in Fos-ir cells in the presence of E2 may be the result of reduced inhibition. Thus, an E2-driven decrease in MnPN activation may lead to a downstream decrease in the VLPO. Additionally, the sex difference in ER alpha expression in the MnPN may account for the difference in sensitivity of males and females to the suppressive effects of E2 on sleep.[8] Further experiments are necessary to determine how ERs may be activated in the MnPN, and any downstream effects triggered, through our necessary-and-sufficient E2 dosing paradigm.

The multiple potential sites of E2 action present an interesting question of how E2 is working to disconnect molecular measures of sleep need from sleep behavior thought to be characteristically directly responsive to that need. We acknowledge that these experiments are heavily focused on the MnPN as one potential key site and that more global actions of E2 within other sleep and arousal circuits may also be important. However, given that we understand so little about how E2 is influencing sleep, expanding these studies to other brain regions and molecular mechanisms in the future could provide more insight into how E2 and sleep networks interact to affect sleep.

2. Estradiol Affects Sleep Time and Slow Wave Activity Differently Depending on Sleep Pressure Conditions

Previously published data suggest that E2 is reducing the total duration of sleep obtained by animals in the dark phase,[7,80] implying the animals should then exhibit a homeostatic rebound in the light phase after this physiological form of deprivation. Our

experiment was therefore designed to test both conditions of physiological sleep pressure (ad libitum sleep) and supraphysiological sleep pressure (sleep deprivation). Our data have shown that E2-treated animals require a lower amount of recovery sleep time following deprivation, suggesting that E2 is either lowering the intrinsic need for sleep or that E2 is changing the sleep mechanisms to alter the recovery sleep response to deprivation.

Quantitative markers derived from EEG outputs exist that can approximate sleep pressure in a reproducible fashion.[5] Sleep intensity, as measured by EEG delta power, also known as slow wave activity, (0.5-4.5Hz) during NREM sleep (NREM-SWA) is a characteristic hallmark of deep sleep need.[129] SWA is generated by synchronized cortical activity during NREM sleep. Increases in the intensity or amount of NREM-SWA is proportional to the amount of prior wake time, and time spent in NREM sleep will dissipate the concentration of SWA. Given these dynamic changes proportional to sleep loss/gain, NREM-SWA is widely used as a reliable marker of sleep homeostasis.[129] Together, our analysis of NREM-SWA suggests that E2 did not greatly dampen the compensatory response to a prolonged sleep deprivation and allowed for deep, homeostatically restorative sleep under those conditions. However, E2 did attenuate baseline homeostatic responses by decreasing NREM-SWA in the light under ad libitum sleep conditions. Thus, our data suggest that E2 may be working in the MnPN to increase the homeostatic set-point for sleep, allowing for a decrease in sleep propensity despite periods of increased wake duration.

Other studies have shown an increase in NREM-SWA over baseline during periods of endogenous high E2 during the proestrus phase of cycling rodents, during the dark phase.[110] This discrepancy with our results in the light phase suggests that the interaction

between the homeostatic and circadian systems regarding E2 requires additional exploration.

3. Estradiol Increases Adenosine Levels in the MnPN

An entire class of molecules known as somnogens have been identified that appear to increase homeostatic sleep pressure, including the nucleoside adenosine, produced in the brain both purposefully and as a waste product of metabolism. Adenosine has been shown to accumulate in the brain, particularly in the basal forebrain, with increasing wake time and decrease in the sleep state, though its dynamics in other brain regions may differ and have been incompletely explored.[149,206-207] While several other molecules have been hypothesized to act in similar fashions to stimulate the sleep pressure homeostat, adenosine is particularly interesting for its dual role as both a marker and a mediator of sleep pressure. If E2 is altering the homeostatic set-point for sleep, one prediction would be that extracellular adenosine would be decreased in the presence of E2. In animals allowed to sleep *ad libitum* in the light phase, extracellular adenosine decreased in both E2- and oil-treated animals, in line with previously published studies of adenosine levels in the preoptic area of cats.[206] However, the decrease in the oil-treated animals was steeper than in E2-treated animals. Under deprivation conditions, E2-treated animals increased their adenosine levels while levels were largely flat in oil-treated animals. These findings add to recent data from other laboratories showing an increase in adenosine during female proestrus[110] and sex differences in adenosine formation.[212]

4. Estradiol Impairs the Ability to Stimulate Adenosinergic Sleep Pressure

The finding that E2 increased MnPN adenosine content while reducing NREM-SWA presents an interesting paradox which suggests that E2 may be working to dampen

the detection of homeostatic sleep need (i.e. adenosinergic signal) and suggests a connection between the effects of E2 and adenosine at the A2AR. Key pharmacological experiments demonstrate that infusion of a highly selective A2AR agonist, CGS 21680 (CGS) into the lateral ventricle of male rats profoundly increases NREM and REM sleep.[151] Other work focusing on the preoptic area sleep active nuclei suggest that activation of A2AR with CGS activates sleep-active neurons in the VLPO[209] and MnPN[151] and increases GABA release in key arousal centers like the TMN.[210-211] To our knowledge, these studies were only done in male rodents and no prior study has directly infused a A2AR agonist into the MnPN, nor treated females with A2AR agonists against differing hormonal backgrounds. Furthermore, a direct link has been shown between estrogens and A2AR, as estrogens have been shown to decrease expression of wake-inhibiting adenosine 2A receptors.[89]

To attempt to resolve the discrepancy between lower sleep time and NREM-SWA and higher preoptic adenosine in E2-treated animals, we designed an experiment to activate the A2A receptor and therefore stimulate the adenosinergic system. We employed a low dose that does not affect sleep *per se* to avoid the direct effect of E2 content on sleep behavior. Our results show that injection of an A2A agonist is sufficient to stimulate sleep in females without E2 treatment, as it is in males. However, when E2 is present in those same animals, even at low levels, the ability of the agonist to stimulate sleep, and thus the ability of activation of the adenosinergic sleep-pressure system to stimulate sleep behavior, is impaired. These results, combined with the sleep behavior and adenosine level data, suggest that E2 is working to disconnect the buildup of adenosinergic sleep pressure from

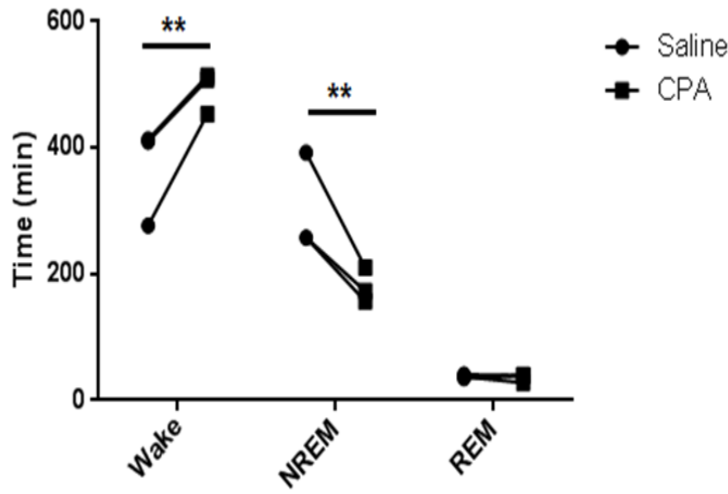
the ability to flip the sleep switch to drive sleep behavior, particularly deep and homeostatically restorative slow-wave NREM sleep.

These data suggest that there is an interplay between estrogen and adenosine which modulates the ability of adenosine to generate sleep pressure, and thus the effect of E2 on wake may be mediated through the adenosinergic system. Further understanding of this interaction could illuminate the relationship between hormonal content and sleep and provide opportunities for treatment of sleep disorders that take female sex and hormonal state into account.

5. Interaction between Two Types of Adenosine Receptors Affects MnPN Sleep Circuits

A2A receptors are not the only sleep-active receptors present in the preoptic area. Preliminary data show that activating the inhibitory A1 receptor specifically at the MnPN leads to an increase in wake time when oil is not on board. Ovariectomized female rats (n=3) were cannulated to the MnPN. 4.5nmol of n-cyclopentyl adenosine (nCPA) or saline vehicle was infused intraparenchymally and sleep was measured for the entire dark phase. After a 7-day washout period, animals received the other treatment. Preliminary data in female rats without E2 show a significant increase in wake time and decrease in slow wave sleep time across the dark phase when nCPA is infused. (Fig. 1)

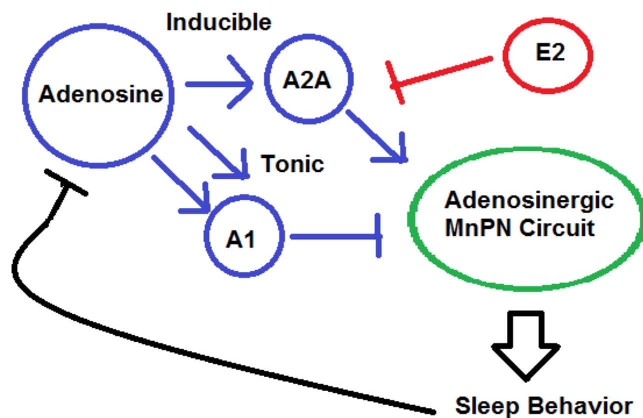
Fig. 54. A1 Receptor Agonist n-Cyclopentyl Adenosine Increases Dark Phase Wake in Ovariectomized Female Rats without E2 Replacement. Female Sprague-Dawley rats (n=3) were OVX and received oil doses in line with our standard paradigm, and cannulated to the MnPN as in the experiments in Chapters II and III. At ZT 11, just before the onset of the dark phase, 4.5nmol n-cyclopentyl adenosine in 5uL saline, or 5uL saline vehicle, was injected into the MnPN via the cannula. n-CPA treated rats showed an increase in wake and a decrease in NREM sleep time across the entire subsequent dark phase (ZT 12-0). (Repeated measure two-way ANOVA main effect of interaction, treatment X sleep state; $F_{(2,6)}=29.01$, $p<.001$; Sidak's multiple comparison test, saline wake vs. n-CPA wake $p<.01$, saline NREM vs. n-CPA NREM $p<.01$)



The finding that A1R activation robustly increases wake in females without E2 on board suggests a potential model for adenosine activation at the hypothalamus. Low concentrations of adenosine, present at all times, activate the A1 receptor in a tonic fashion. As sleep pressure builds throughout wake time, adenosine concurrently rises to a level that activates the stimulatory A2A receptor. At high levels of adenosine, the A2A receptor overcomes the A1-mediated tonic inhibition and activates the sleep-active preoptic circuitry, inhibiting downstream wake activation and flipping the sleep switch. Our data suggest that E2 may be working to inhibit the activating A2A circuit even at high levels of adenosine. This process allows the tonic inhibition of sleep drive from A1 to dominate and reduces sleep time in times when the homeostat is mildly stressed. However, under conditions of high sleep need, building sleep pressure eventually overcomes the E2-mediated inhibition and activates the A2AR, flipping the sleep switch at a greater level of

adenosine. Our NREM-SWA data suggests that the E2-treated animals are less efficient at resolving baseline homeostatic need once they do enter the sleep state, which would suggest a reason for why sleep times to not differ under conditions of physiological sleep pressure in spite of the homeostat being dominated by the wake-promoting A1 circuitry. This model therefore encompasses both the pharmacological data of E2-adenosine interaction and the greater adenosine levels in E2-treated animals. These findings could suggest a potential pathway for the interaction between E2 and the adenosinergic system (Fig. 55).

Fig. 55. Speculative Schematic of Proposed Mechanism for E2-Adenosine Interaction.



Adenosine at the MnPN may act through tonic activation of sleep-inhibiting A1 receptors at all times **and** inducible activation of sleep-promoting A2A receptors during times of high adenosine levels (high sleep pressure). E2 may inhibit the activation of A2A receptors, causing sleep-inhibitory A1 tone to dominate at higher adenosine levels, and raising the sleep pressure and adenosine threshold needed to induce sleep behavior.

6. Other Potential Sites and Mechanisms Exist for Estradiol Suppression of Sleep

Sleep-wake behaviors are regulated by reciprocal connections between sleep-promoting nuclei in the preoptic area (POA) and arousal centers in the hypothalamus and brainstem.[84] The ventrolateral preoptic area (VLPO) and the median preoptic nucleus (MnPN) are two key sleep-active nuclei involved in the onset and maintenance of sleep. Both regions express Fos, a proxy marker for neuronal activation, during sleep periods, which correlates inversely to the amount of previous sleep.[84, 160, 213-214] Thus, the neuronal

activation of these regions can be seen as a proxy for sleep pressure, or homeostatic sleep need. Moreover, the VLPO has long been shown to have sensitivity to changes in ovarian steroids. E2 replacement following ovariectomy reduces both Fos expression and protein expression of lipocalin-type prostaglandin D synthase (L-PGDS), the synthesizing enzyme for the somnogen prostaglandin D2, within the VLPO of female rats compared to ovariectomized controls.[7] These POA nuclei are also proposed to modulate homeostatic sleep drive. Together, these data suggest that E2 alters critical factors involved in sleep, particularly in the sleep-active POA.

Our findings are the first report of direct actions of E2 on sleep behavior within a sleep-active nucleus. It is clear, however, that the MnPN is only one region mediating E2 suppression of sleep. Local ER antagonism in the MnPN only partially rescued baseline sleep and wake. Therefore, it is likely that E2 has direct actions in other nuclei regulating sleep/wake. For example, the histaminergic neurons in the tuberomammillary nucleus may be targets for E2. Histamine is involved in arousal and is a direct downstream target of the VLPO.[213-215] Fos expression is higher in ovariectomized females treated with E2 compared to oil, indicating increases in arousal following E2 treatment.[7] It is unclear if this is a direct or indirect effect of E2. Nuclei within the ascending reticular activating system contain ERs and are potential target sites for E2-mediated arousal.[216-221] The noradrenergic neurons of the locus coeruleus (LC) are involved in arousal and contain binding sites for E2, suggesting that E2 may directly increase arousal by increasing activation of the LC. Additionally, the cholinergic neurons of the basal forebrain and pedunculopontine tegmental nucleus/laterodorsal tegmental nucleus, which play a key role in cortical activation during arousal, can also be target sites for E2.[222] Increased activation of these

regions by E2 may account for increased high frequency oscillations in sleep EEGs, which may be an objective measure of poor sleep quality.[223]

Alternatively, E2 may act within other sleep-promoting nuclei like those involved in REM sleep generation. It is possible that E2 either directly or indirectly reinforces the activity of REM-OFF cells in the brainstem.[224] MCH/GABAergic neurons are proposed to control the switch into REM sleep by inhibiting REM-OFF cells.[225] MCH neurons do not express ER;[226] however, the neighboring GABAergic population in the lateral hypothalamus may do so. E2 may reduce the activity of MCH/GABAergic cells (directly or indirectly) to suppress REM sleep.

Beyond the question of a site of action, the question of how, with regards to molecular and neurological mechanisms, E2 may be meditating sleep effects is an important one. Several potential mechanisms could exist for E2-adenosine interactions. An obvious potential avenue of investigation is the direct effect of E2 at the adenosine receptor, with nuclear signaling of E2 potentially directly affecting expression in the MnPN, as has been shown in other preoptic regions[89] or changing the trafficking of A2AR components to deactivate them. Adenosine microenvironmental homeostasis may also be a potential mechanism of E2-adenosine interaction. Over the past two decades, glial cells have been increasingly recognized as critical players in the regulation of sleep and sleep homeostasis. More specifically, gliotransmission, (i.e. vesicular release of neuro- modulators and transmitters) of ATP/adenosine has been shown to modulate sleep pressure.[227] Optogenetic stimulation of astrocytes in wake-active nuclei, such as the TMN, increase sleep time and intensity.[228-229] Past work has demonstrated that preoptic area/hypothalamic astrocytes are exquisitely sensitive to E2. Therefore, it is possible that E2 is acting through astrocytes to

decouple adenosine levels from signaling activity. E2 may also be acting entirely outside of the adenosinergic system through modulation of other somnogen mediators, such as L-PGDS,[87-88] or through activation of wake- promoting systems. Additionally, the glymphatic system has been shown to be important for clearance of metabolites and other waste products from the brain, and is upregulated by as much as a factor of ten in the sleep state.[96] While adenosine as a small molecule and not a protein is likely not predominantly cleared through glymphatic flow, the hypothesis that sleep serves in part as a process of clearing products of cellular metabolism could suggest that E2 is impacting the clearance of adenosine, potentially through enzymes like adenosine deaminase, leading to a tolerance effect of higher adenosine levels in E2-treated rodents.

7. Estradiol-Sleep Interactions Have Deep Clinical Relevance

Women have been consistently diagnosed with insomnia and other sleep disorders at a markedly higher rate than men.[5] This disparity in humans may be due to psychosocial factors such as a higher presence of anxiety in females.[5,62] However, there is strong evidence that hormonal complement plays a role; most strikingly, the sex difference in sleep quality emerges in females with puberty[63] and disappears at menopause. Sex hormones have been shown to interact with the sleep-wake system in both the basic and clinical literature, and women and men have long been clinically shown to have differing sleep patterns.[5] however, studies of different sleep patterns between the sexes in humans paint a contradictory picture to the clinical findings. In particular, women paradoxically sleep longer than men, but generally self-report a lower sleep quality.[5] Objective data suggest women should have higher sleep quality than men; women have longer total sleep time and less total wake time, a shorter latency to sleep onset, and higher sleep efficiency

than men.[58-59] EEG studies have also shown a higher proportion of deep slow-wave sleep (stage 3) and less light sleep (stage 1 and 2) in women than men.[60] When considered in conjunction with our findings from rodents, these data could give credence to a connection between estrogens and sleep in both rodents and humans wherein E2 serves to decouple physiological measures of sleep need from sleep behavior. It is possible that an analogous mechanism may be in play for women: sleep times and stages that would be sufficient for resolution of homeostatic need without interference of hormones like E2 could become insufficient at fully resolving women's homeostatic need when E2 is present, leading to subjective sleep complaints. Our data showing E2 serves to change the relationship between homeostatic sleep need and sleep behavior could in part explain the discrepancy between those objective and subjective findings.

B. Conclusion

The understanding of the role played by sex hormones as biological factors in sleep regulation may potentially offer a deeper insight as into why women are at a significantly higher risk for insomnia and sleep disruption[1]. Work in both rats and mice has demonstrated that in the absence of circulating gonadal sex steroids, sex differences in sleep behavior and architecture are eliminated,[76-77.79-80] suggesting that sex differences in sleep are primarily dependent on circulating sex steroids. Furthermore, our previous work in adult female rats demonstrates that sleep-wake behavior and activation of the preoptic area sleep nuclei are highly sensitive to fluctuations in circulating E2.[7,80] Using a rat model of exogenous replacement of E2 that mimics the endogenous cyclic fluctuations of the estrous cycle, we have extended our findings about the relationship between E2 and homeostatic sleep pressure. Our studies have demonstrated the MnPN is necessary and sufficient for

E2-mediated sleep suppression. We further show that E2 has different effects on sleep time and NREM-SWA, a measure of the homeostatic efficiency of sleep, under conditions of normal sleep and sleep deprivation. E2 serves to decrease NREM-SWA but not sleep time, in the light phase under normal sleep, while in recovery E2 decreases sleep time but not NREM-SWA following deprivation. These differences suggest that E2 has effects that interact with the homeostatic sleep pressure system. We further found that E2 increases levels of extracellular adenosine, a measure of homeostatic sleep need, under both normal and deprivation sleep conditions. To resolve the discrepancy between the decrease in behavioral markers associated with homeostatic sleep need and increase in levels of adenosine, we employed an agonist of the A2A receptor to stimulate the adenosinergic sleep pressure system. These studies show that E2 blunts the ability of the A2A agonist to drive sleep behavior, showing an interposition of E2 into the sleep pressure system that blunts the ability of adenosine to act as a somnogen. Overall, these experiments show an interaction between E2 and adenosinergic homeostatic sleep pressure at the MnPN. Further understanding of these mechanisms and circuitry could open new avenues to understanding and treatment of sleep disorders in both women and men that takes steroid profile and biological sex into account.

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