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

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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.

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. 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^{o} 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).

5. 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).

6. 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.

7. 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^{o}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^{o}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.

8. 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).

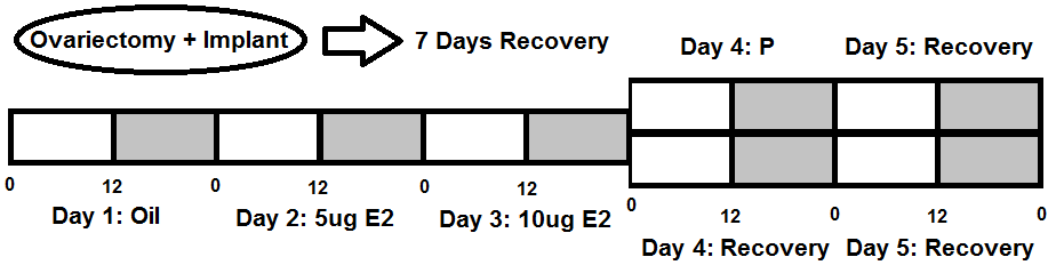


Fig. 7. Timeline of Hormonal Recapitulation Experiment. Ovariectomized Female Sprague-Dawley Rats (n=17) were administered an oil injection on Day 1, 5ug of E2 on Day 2, and 10ug of E2 on Day 3. On Day 4, the animals were split into two groups, with one group being administered progesterone to mimic 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.

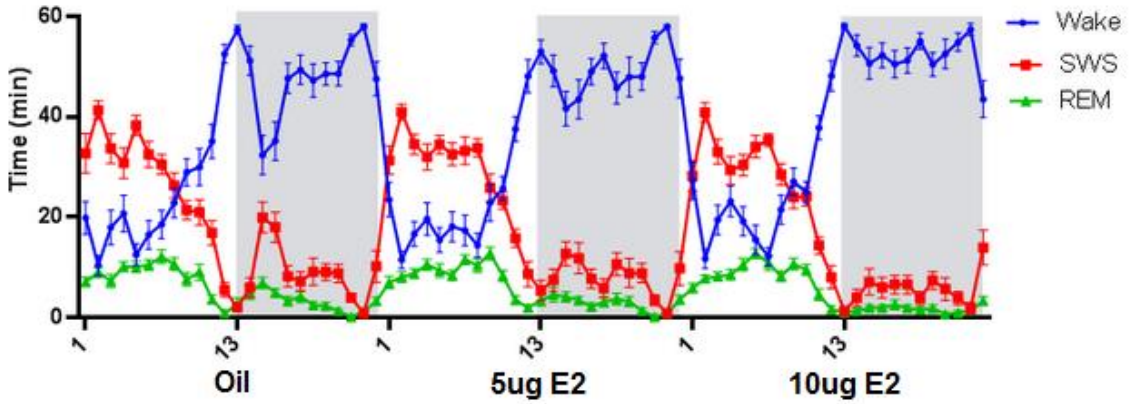


Fig. 8. Proestrus-level E2 Suppresses Sleep and Increases Wake. Using a within animal design, we recorded EEG/EMG data from OVX adult rats treated with our standard dosing paradigm of 2 injections of E2 24 hours apart. On the day of high-dose E2 administration (Day 3), mimicking proestrus hormone levels, 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)

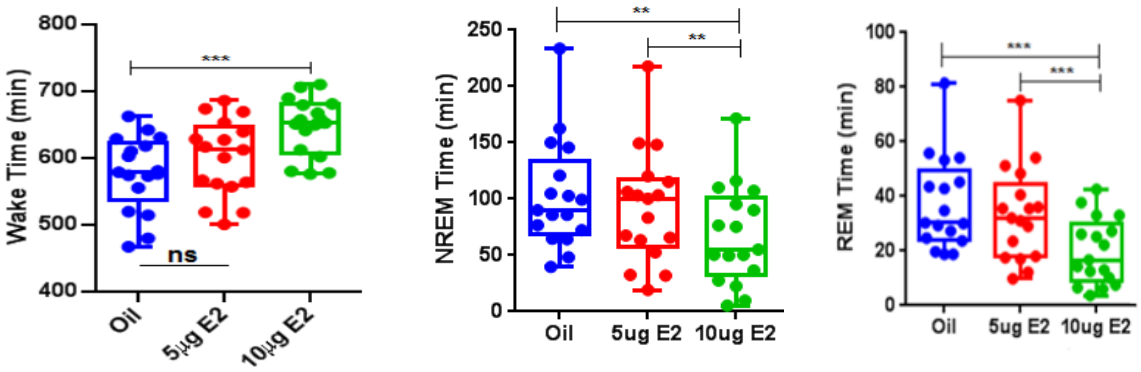


Fig. 9 A-B-C. E2 Increases Total Dark Phase Wake Time and Decreases Total NREM and REM Sleep Time. **(A)** In the dark phase, animals showed a significant increase in wake time relative to oil on the day of high-dose E2 treatment (p=.0005). The low-dose E2 treatment did not have a significant effect 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)

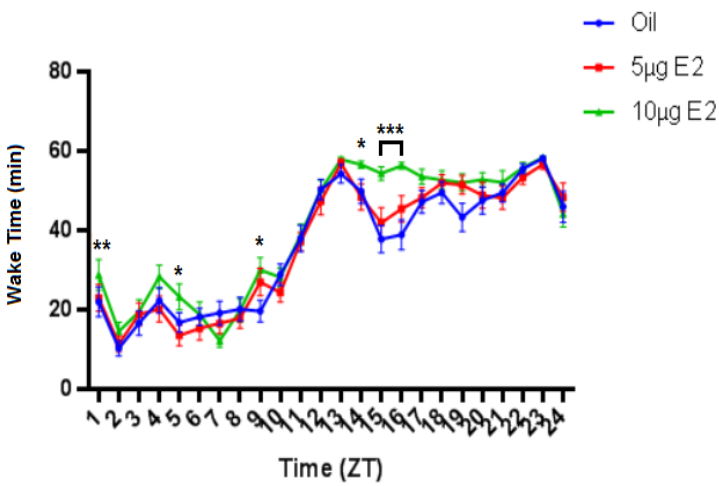


Fig. 10. E2-mediated Sleep Suppression is Most Prevalent in the Early Dark Phase. Analysis of the sleep time by hour across the treatment days, shows that wake time is not significantly different between the oil and low dose E2 at any treatment time. However, the high-dose E2 treatment showed a significantly 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 $p<.001$)

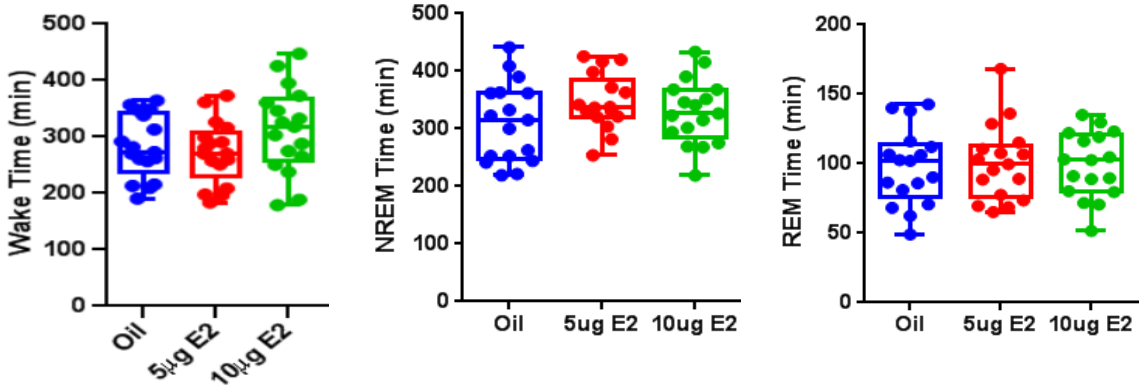


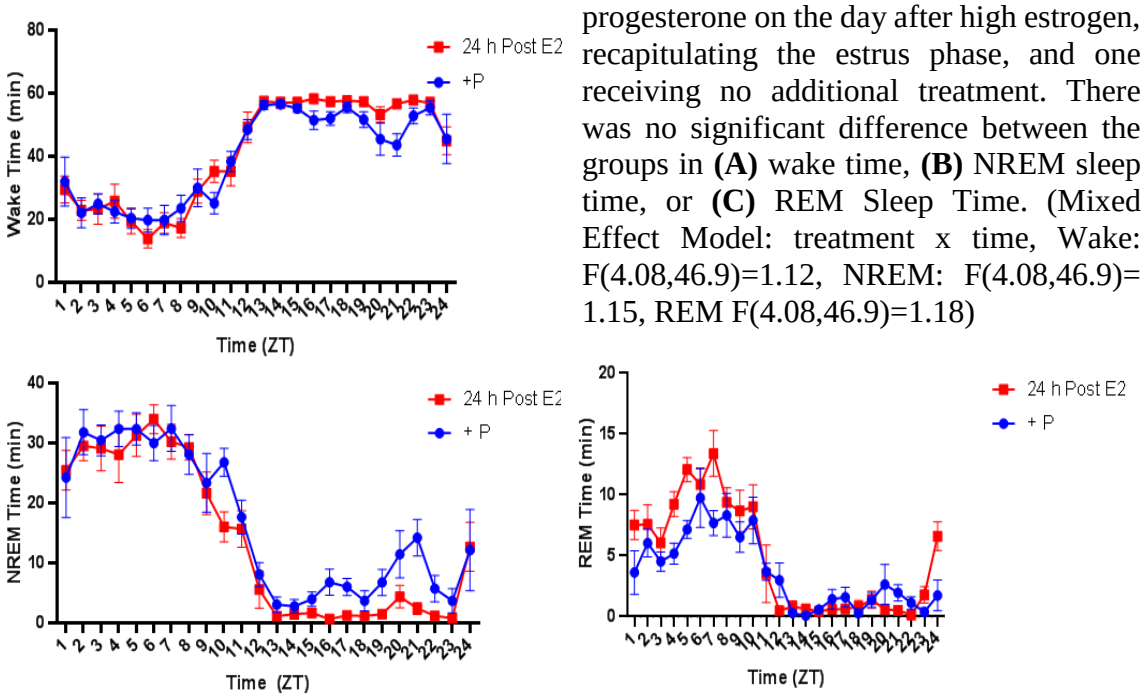
Fig. 11. E2 does not Affect Total Sleep or Wake Time in the Light Phase. There is no significant difference in **(A)** wake time, **(B)** NREM sleep duration, or **(C)** REM Sleep Duration across any treatment.

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.

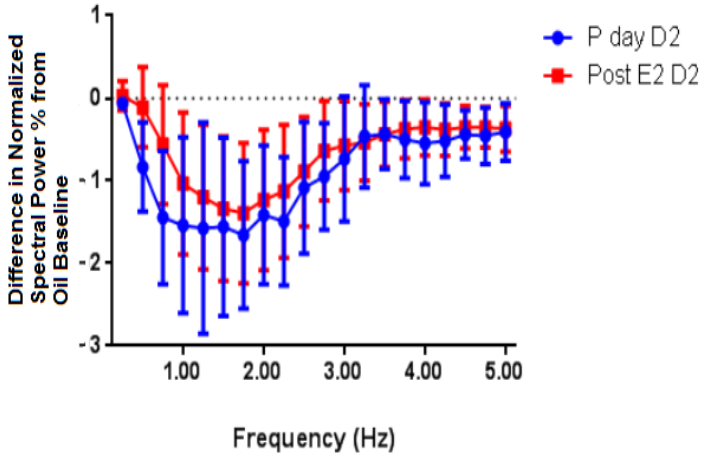


Fig. 13. Progesterone Produces No Change in EEG Spectral Power. We compared power spectra of the progesterone and non-progesterone treated groups on Day 4 relative to their own Day 1 oil baseline. In both groups, we see a decrease in spectral power on the day of progesterone treatment, with no significant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

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.

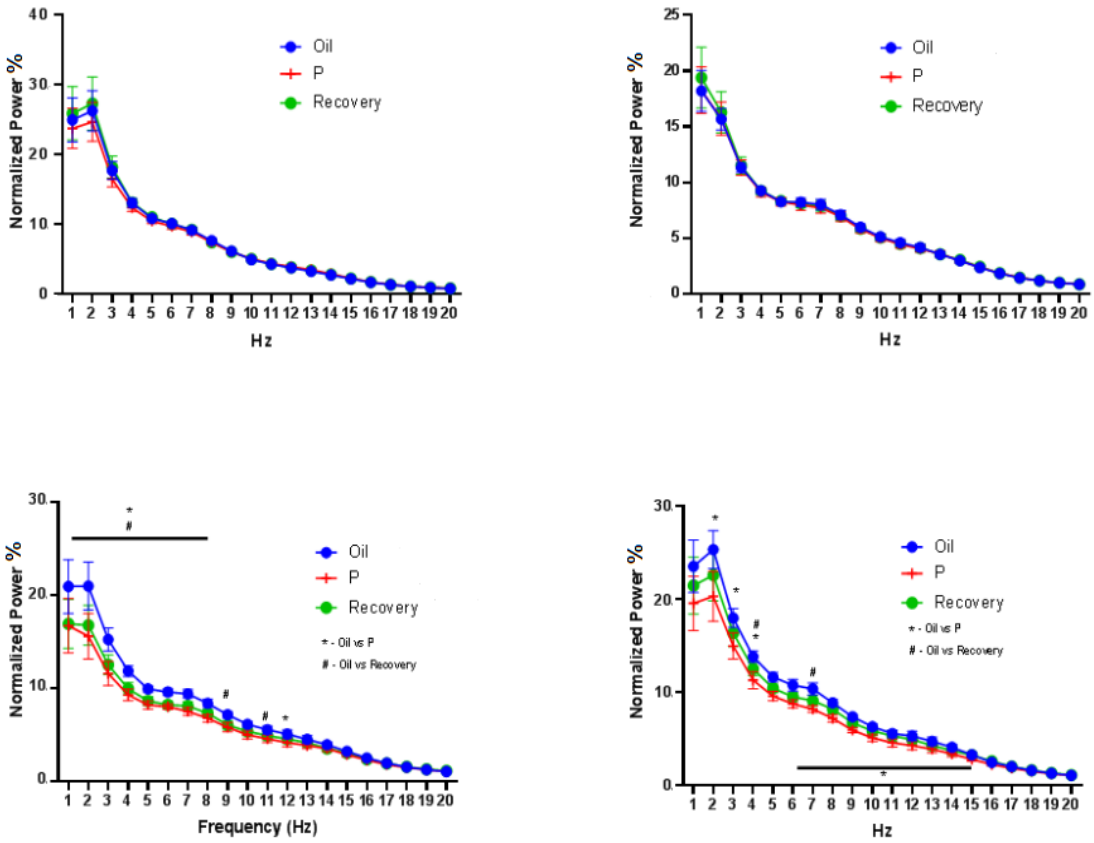


Fig. 14 A-B-C-D. E2 Decreases Spectral Power in the Dark Phase, Particularly in the Delta Band. We examined the spectral power across all animals on Day 1, the day of oil treatment (Oil), Day 4, the day of progesterone treatment (P) following E2, or the following Day 5 (Recovery). (See Fig. 7) There 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, $F(57, 580) = 1.901$, $p<.001$)

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.

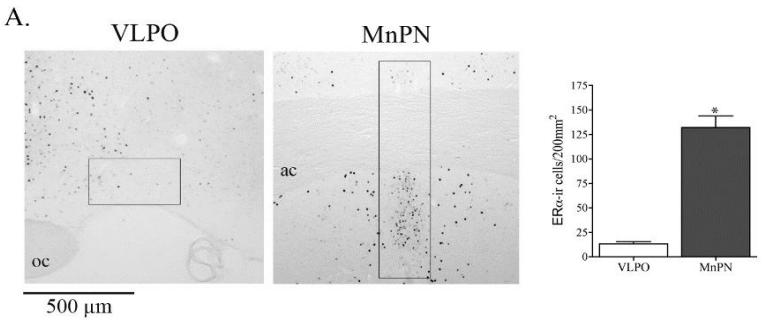


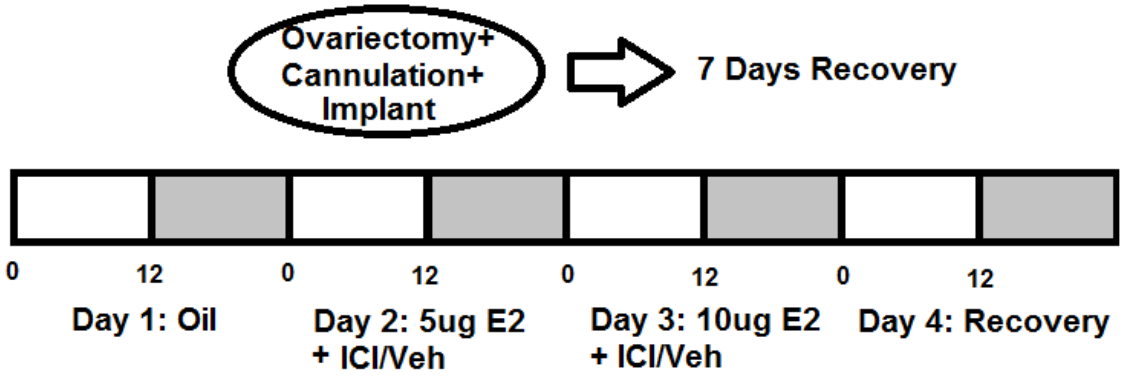
Fig. 15. (Figure from DMC) Estrogen Receptors are Highly Expressed in the Female MnPN. Staining for Estrogen Receptors (ER) show there is a high concentration in the MnPN but not the VLPO of females. (Student's *t*-test, *, $p<0.05$ vs. VEH).

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).

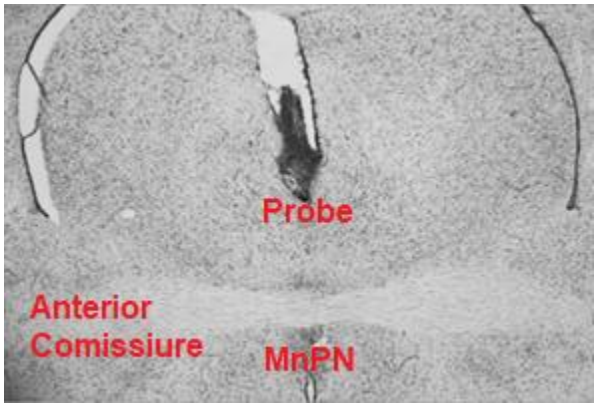


Fig. 17. Representative Image of MnPN Probe Placement. Probe placement to the MnPN was confirmed histologically.

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).

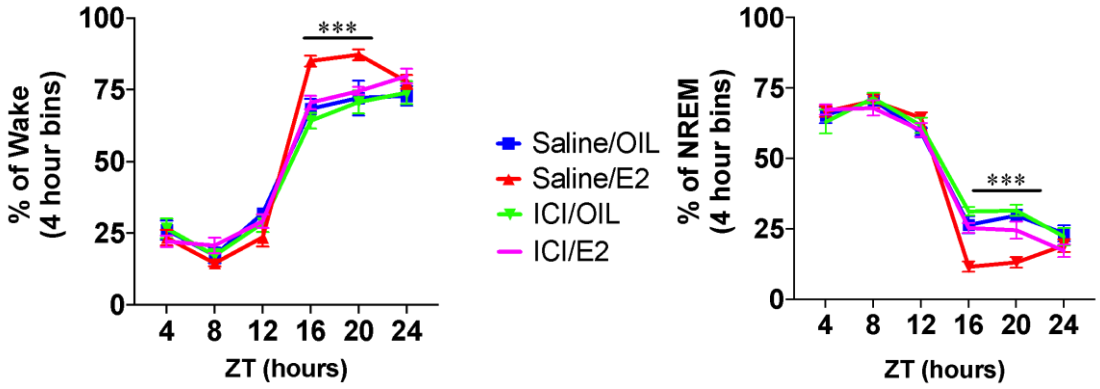


Fig. 18 A-B. ER Antagonist ICI Reduces Wake Time and increases NREM Sleep Versus E2 Replacement. (A) Injection of ICI against a background of E2 treatment reduced wake time across much of the dark phase, with statistically significant decreases from ZT 12-16 ($p<.001$) and ZT16-20 ($p<.001$). There was 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).

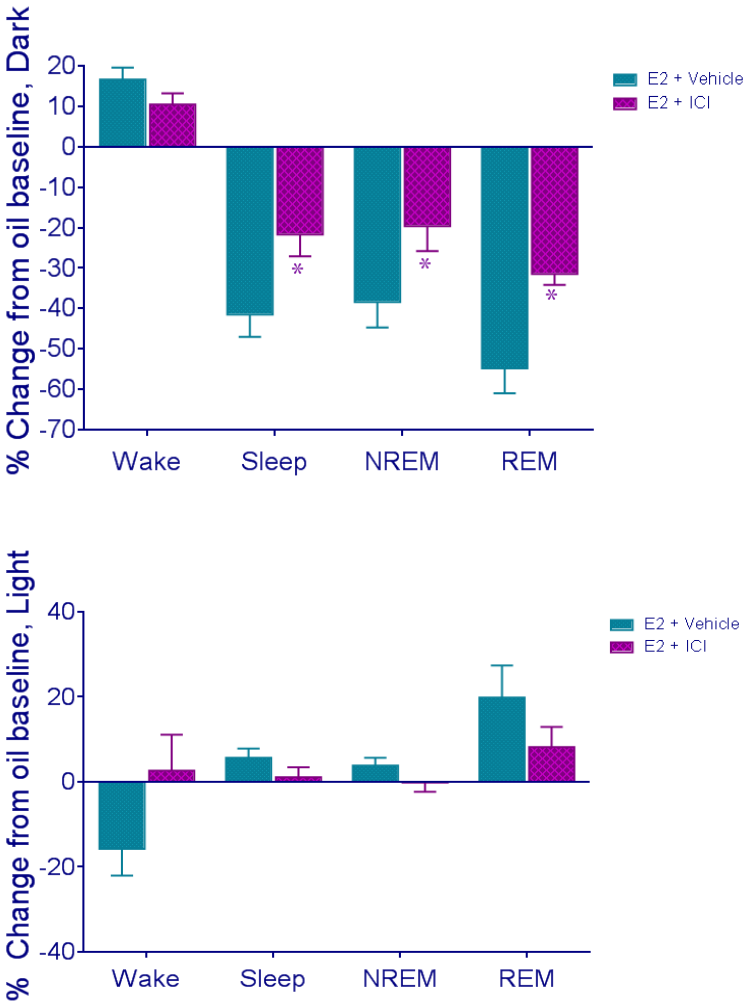


Fig. 19. ICI Treatment Partially Rescues E2-Mediated Dark Phase Sleep Suppression. We also compared total sleep time relative to each animal's oil baseline recording, both with E2 treatment and vehicle and E2 and ICI treatment. **(F)** During the Dark Phase, ICI treatment partially rescued the E2-mediated 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.

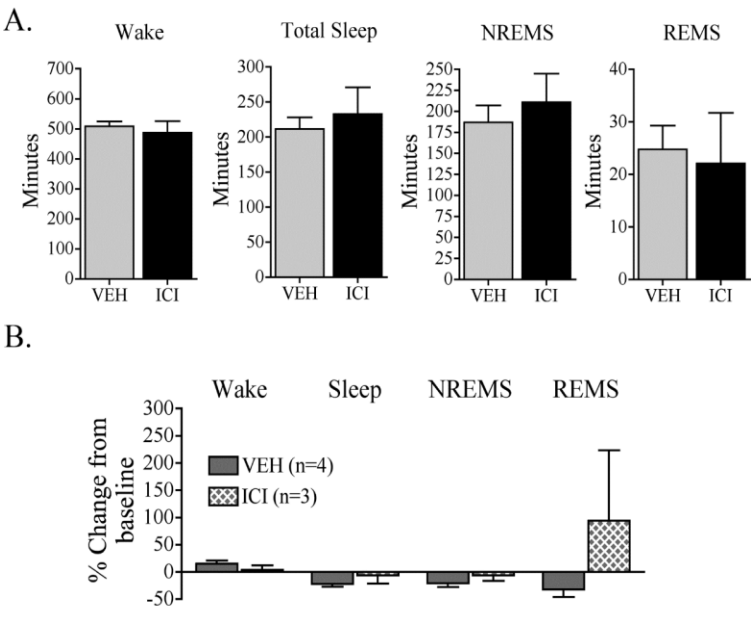


Fig. 21 A-B. (Figure from DMC) ICI Infusion to the VLPO does not Change E2-Driven Sleep Behavior. Unlike infusion into the MnPN, infusion of ICI into the VLPO did not affect wake time, total sleep time, or REM sleep time, either **(A)** in overall time or **(B)** change from oil control baseline. The 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).

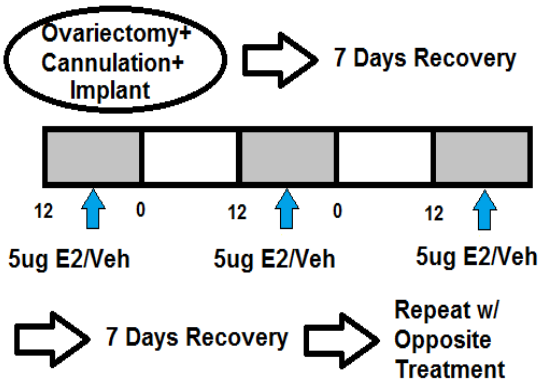


Fig. 22. Timeline of Direct E2 Infusion Experiment. Female Sprague-Dawley rats (n=9) were ovariectomized and implanted with EEG/EMG telemeters and guide cannula to the MnPN. After recovery, animals were infused at ZT18 with 5ug cyclodextrin- encapsulated E2 in 5uL sterile saline, or 5ug cylodextrin vehicle 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.

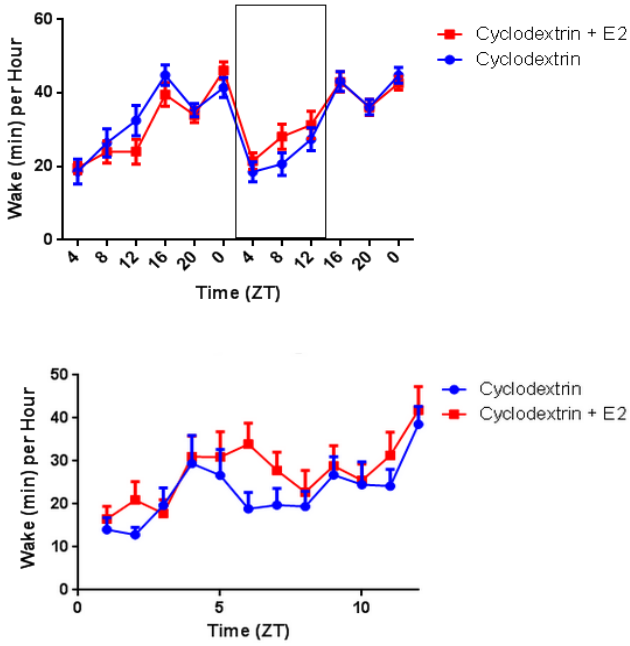
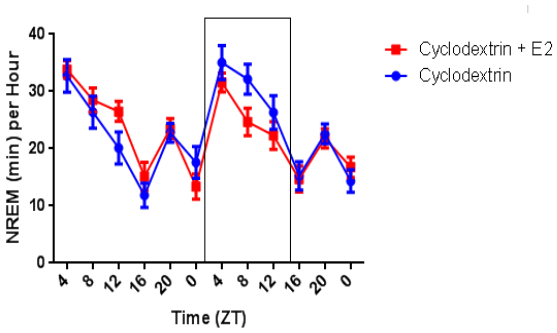
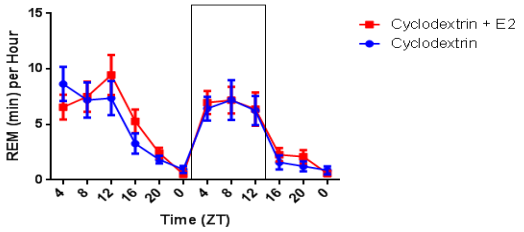
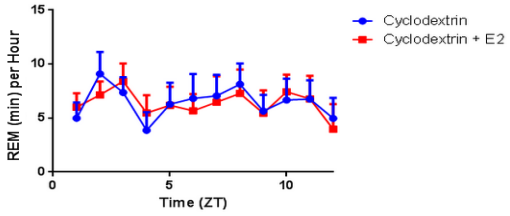


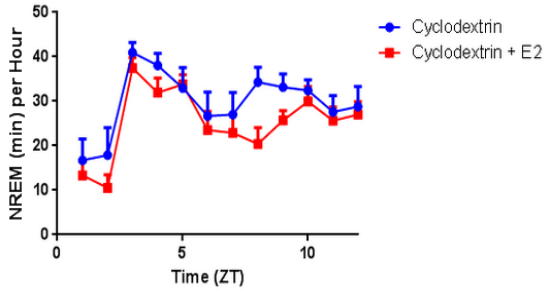
Fig. 23 A-B. Direct E2 Infusion Increases Dark Phase Wake. E2-treated animals showed an increase in wake time in the 2nd light phase (p=.03, two-way ANOVA, main effect from ZT0-12 on treatment day 2). Lower Panel is increased detail of boxed region.

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)

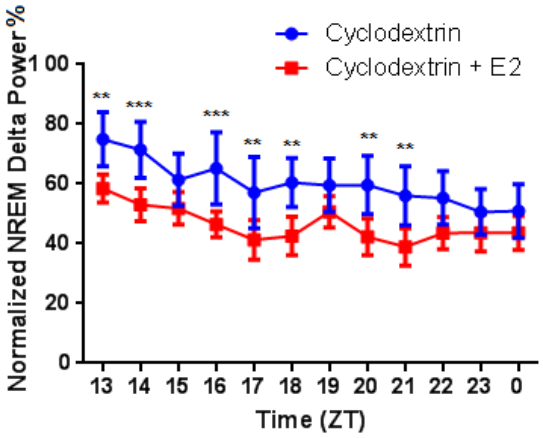


Fig. 26. E2 Infusion Decreases NREM Delta Power. We analyzed the spectral power with and without E2. NREM Delta Power from E2-treated animals shows a significant decrease in several time points in the second light phase relative to oil controls. (Repeated Measures two-way ANOVA, main effect of hormone, F (1, 9) = 3.228P = 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.

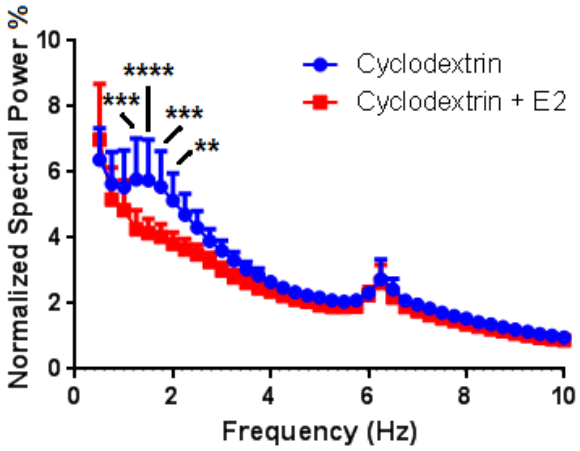


Fig. 27. Direct Infusion of E2 Changes Power Most Significantly in the Low Delta Band. Spectral Fourier analysis revealed the decrease in delta power was localized to a particular portion of the low delta band, with the difference between E2 and vehicle significant at the 1.25 ($p<.001$), 1.5 ($p<.0001$), 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.

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