EFFECTS OF OVARIAN HORMONES ON SLEEP AND RECOVERY FROM SLEEP DEPRIVATION IN OVARIECTOMIZED MIDDLE-AGED FEMALE RATS

by

Margaret Elizabeth Seary

Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
June 2011

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DALHOUSIE UNIVERSITY

DEPARTMENT OF ANATOMY AND NEUROBIOLOGY

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DALHOUSIE UNIVERSITY

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DEPARTMENT OR SCHOOL: Department of Anatomy and Neurobiology

DEGREE: MSc CONVOCATION: October YEAR: 2011

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DEDICATION PAGE

I would like to dedicate this work to 2 "individuals". First and foremost, I would like to both thank and dedicate my thesis to Bill Binsfeld, he is my rock; he holds me up and stabilizes me. He has always believed in me, even when I couldn't, provided levity and distractions when I needed them, and provided the thumbscrews when I needed to be forced back to work. Secondly, I would like to dedicate my thesis to Babs, my dog, who could always make me laugh, no matter how gloomy my mood, and who could always convince me to take a little time to play.

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ABSTRACT

Menopausal symptoms, including sleep problems, occur as a result of reduced production of ovarian hormones in middle-aged women, and are often treated with replacement of these hormones. However, the efficacy of hormone replacement for improving sleep is controversial. We assessed sleep/wake patterns during baseline and recovery following 6 h of sleep deprivation in ovariectomized middle-aged rats treated with oil, estradiol, or estradiol and progesterone. We found that, at baseline, hormone administration reduced rapid eye movement (REM) sleep initiation and non-REM sleep amount, promoting wakefulness, particularly during the dark (active) phase, but that, during recovery following sleep deprivation, hormonal treatment reduced sleep intensity initially and lengthened REM sleep recovery. These results indicate that in middle-aged female rats ovarian hormones modulate baseline and recovery sleep differently, possibly by modulating circadian and homeostatic regulation of sleep in an age-dependent manner.

LIST OF ABBREVIATIONS USED

5-HT	Serotonin
ACh	Acetylcholine
ANOVA	Analysis of variance
BF	Basal forebrain
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CREB	cAMP response-element binding
DA	Dopamine
DR	Dorsal raphe nucleus
E	Estrogen
EEG	Electroencephalogram
EMG	Electromyogram
EPSP	Excitatory postsynaptic potential
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FFT	Fast-Fourier transform
GABA	γ-aminobutyric acid
Glu	Glutamate
HA	Histamine
HE	High estrogen dose
HRT	Hormone replacement therapy
i.p.	Intraperitoneal
IPSP	Inhibitory postsynaptic potential
LC	Locus coeruleus
LDT	Laterodorsal tegmental nucleus
LE	Low estrogen dose
LEP	Low estrogen and progesterone dose
LH	Lateral hypothalamus
LP	Low progesterone dose
LPT	Lateral pontine tegmentum
LSD	Least significant differences
LTP	Long-term potentiation
MnPO	Median preoptic nucleus
MR	Midbrain raphe nucleus
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NREM	Non-REM
NREMS	NREM sleep
NT	Neurotransmitter
OVX	Ovariectomy
P	Progesterone

PAG	Periaqueductal gray
PB	Parabrachial nucleus
PC	Precoeruleus nucleus
POA	Preoptic area
PPT	Pedunculopontine tegmental nucleus
PR	Progesterone receptor
REM	Rapid eye movement
REMS	REM sleep
s.c.	Subcutaneous
SCN	Suprachiasmatic nucleus
SD	Sleep deprivation
SLD	Sublaterodorsal region
SWS	Slow wave sleep
TMN	Tuberomammillary nucleus
vlPAG	Venterolateral periaqueductal gray
VLPO	Ventrolateral preoptic nucleus
vPAG	Ventral periaqueductal gray
VPOA	Ventral preoptic area
ZT	Zeitgeber time

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Dr. Kazue Semba; she has always been willing to take time to answer questions and has happily acted as a sounding board for ideas whenever I needed one. I have popped by her office whenever the fancy struck, and she has always graciously taken time to help me out with whatever was on my mind.

Samuel Deurveilher has also been there to help and support me through all the stages of my thesis; I can honestly say that there would have been many times when I would have been well and truly stuck without him. As our resident expert, Sam taught me surgical techniques and experimental protocols, he has guided me through sleep software (which is not fun, let me assure you) and statistical analyses, and he has personally spent an inordinate amount of time double checking sleep-wake scoring. Thank you Sam!

I have been very fortunate to be surrounded by wonderful individuals, who made every day in the lab a little bit better. To my compatriot in arms, Chantalle Briggs, who has listened to me whine and cheer (usually not at the same time), who offered a helping hand whenever she could, who *willingly* proofed my thesis (and other assorted documents), and with whom I have shared many meals and refreshing beverages! Do not worry Chantalle you will not be forgotten. To Joan Burns, who taught me surgical techniques, helped with scoring data and always provided a sympathetic ear when I needed to vent my frustrations or share my joys. I appreciate all the time we've spent together. To Erik, who shared my enthusiastic interest in hormones; thank you for your help and all of our productive (and not so productive) conversations. To the lab members,

Alex Madore, Kristin Ko and Katie Miller, who were there to chat, to help with sleep deprivations and to help with scoring. Thank you all.

My friends in the community have been there every step of the way; they provided help and distractions in equal measure, both of which helped keep me balanced and productive. Thank you Anna for, well for everything! If I listed all the things I need to thank you for my acknowledgements would be longer than the rest of my thesis, so I will leave it at a general thank you and hope you know how important you are. To Elke thank you for all the dog walks and Ray for the deck building adventures. To Katerina and Jenn, i want to thank you for always being there when I need you.

To my family who have suffered silently, most of the time, as I prattle away. To my mother, Susan, who always supported me and who never failed to show how proud she was of me. To my father, Richard, who has always supported me in my academic pursuits. To my sister, Alison, who may not always understand what I'm talking about, but who smiles and nods with the best of them and who can always make me laugh - I miss our Sunday dinners. To my sister, Judith, who has always been there with help and advice whenever I am in need. And to the Binsfelds, and Gwen in particular, who have listened, provided support, and celebrated with me; you are my second family, thank you!

To Babs for your unconditional love and adoration, you will always be a puppy to me. And last, but never least, I want to thank Bill. You have always showed a tremendous amount of faith in me, without which I am not sure whether I could have believed in myself. You have hated or disliked people on my behalf, which left me free to forgive them. You have always been there in equal measure for the good, the bad, and the ugly. You mean the world to me and I hope you never forget it. I love you.

CHAPTER 1 - INTRODUCTION

1.1 SLEEP

1.1.1 Introduction to Sleep

Chronic sleep restriction is common in industrialized countries as a result of a rising demand for people to work extended hours and at non-traditional times (Broman et al., 1996; Hurst, 2008). Chronic sleep loss has been associated with an increased risk of cardiovascular disease, obesity and diabetes (Knutson & Van Cauter, 2008). Sleep loss and circadian disturbances have an enormous economic impact related to workplace absenteeism, increased accident risk and treatment costs (Daley et al., 2008). Women report more sleep problems than men (Moline et al., 2004) and aging, in both sexes, is associated with decreased sleep quality and quantity (Dijk & Duffy, 1999). The quality of recovery sleep following sleep loss reflects how effectively people compensate for sleep loss, but little is known about how aging and female sex hormones affect the recovery of lost sleep.

Sleep, in mammals, alternates regularly with waking, and is comprised of two major states: rapid eye movement sleep (REMS) and non-REM sleep (NREMS). REMS is characterized by low-amplitude, moderately high-frequency electroencephalogram (EEG) activity (4.5-8 Hz; theta waves), postural muscle atonia, and frequent REMs, and is associated with dreaming in humans. NREMS is divided into 3 different stages, which are characterized by increasing amounts of high amplitude, low-frequency EEG activity, as well as other specific features in the EEG. The deepest stage of NREMS (stage 3), also called slow-wave sleep (SWS), is characterized by large, continuous, slow EEG waves (0.5-4 Hz; delta waves) (Lydic & Baghdoyan, 1999; Moser et al., 2009).

Circadian (about a day) and homeostatic processes interact to regulate the timing and amount of sleep. Circadian rhythms are primarily controlled by the suprachiasmatic nucleus (SCN) in the hypothalamus in mammals. Neurons in the SCN fire rhythmically due to the intrinsic molecular clock housed in these cells and serve as the pacemaker to influence the timing of many behavioural and physiological processes, including sleep. Homeostatic mechanisms strive to maintain stability in physiological processes through feedback from the processes they regulate. Compensatory responses to sleep loss are driven by homeostatic mechanisms and result in increased sleep drive, intensity and duration (Borbély, 1982; Borbély & Tobler, 1985; Achermann & Borbély, 2003; Mistlberger, 2005).

Acute sleep deprivation (SD) results in a compensatory rebound increase in both sleep duration and the amount of EEG slow-wave activity (1-4 Hz) during NREMS. NREMS slow-wave activity is a well established measure of sleep intensity that reflects previously accumulated sleep need (or debt); it rises progressively as a function of the duration of both spontaneous and enforced waking, and decreases progressively during sleep (Borbély & Achermann , 1999; Tobler, 2005). An increase in theta wave frequency (4.5-8 Hz) during waking has also been suggested to be a marker of accumulating sleep need during prolonged wakefulness (Finelli et al., 2000; Vyazovski & Tobler, 2005; Wigren et al., 2009).

Sleep/wake states, and the regulation of transitions between states are controlled by nuclei dispersed throughout the brain, which use several neurotransmitter (NT) systems. Wake-promoting projections arise from neurons located in the upper brainstem. Cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei provide

inputs to the thalamus (Satoh & Fibiger, 1986; Hallanger et al., 1987). In contrast, monaminergic and glutamatergic neurons arising from the locus coeruleus (noradrenaline; NA), parabrachial nucleus (glutamate; Glu), precoeruleus nucleus (Glu), dorsal raphe nucleus (serotonin; 5-HT), and ventral periaqueductal gray (dopamine; DA) provide direct inputs to the basal forebrain, hypothalamus and cerebral cortex. All brainstem arousal-promoting pathways can also inhibit sleep promoting neurons in the ventrolateral preoptic area (Steininger et al., 1999; Hur & Zaborszky, 2005; Kocsis et al., 2006; Lu at al., 2006a; Lu et al., 2006b). Wake-promoting systems also arise in the hypothalamus, including the tuberomamillary nucleus (histamine; His) and orexin neurons, which reside in the lateral hypothalamus. Orexin neurons directly innervate the cerebral cortex and the basal forebrain; in addition, they support activity in the brainstem arousal pathways (Peyron et al., 1998; Adamantis et al., 2007; Carter et al., 2009). A summary of wake-promoting pathways is shown in Figure 1 and Table 1.

Sleep-promoting neurons are primarily located in the ventrolateral and median preoptic nuclei and inhibit the wake-promoting nuclei in the hypothalamus and the brainstem, using the NT gamma-aminobutyric acid (GABA), and, in case of ventrolateral preoptic nucleus neurons, galanin (Sherin et al., 1996; Sherin et al., 1998; Lu et al., 2000; Suntsova et al., 2002; Gong et al., 2004). Transitions between NREMS and REMS are controlled, in part, by reciprocal inhibitions of the following brain areas: the ventrolateral periaqueductal gray (GABA), lateral pontine tegmentum (GABA), sublaterodorsal region (GABA; Glu), locus coeruleus (NE), dorsal raphe (5-HT), laterodorsal tegmental nucleus (acetylcholine; ACh), pedulopontine tegmental nucleus (ACh), venterolateral preoptic

area (GABA), and parabrachial nucleus (Glu) (Saper et al., 2010). A summary of the sleep-promoting pathways are shown in Figure 2 and in Table 2.

1.1.2 Age Associated Changes in Sleep Patterns

With increasing age there is a decline in sleep quality, which manifests as decreased SWS duration and intensity; deficits in sleep quality negatively impacts an individual's overall sense of quality of life (Dijk & Duffy, 1999; Carrier et al., 2001; Gaudreau et al., 2001). The circadian regulation of sleep and wake is also altered: older people tend to sleep and wake at earlier phases of their circadian cycle compared to young individuals. Most of these changes begin during early middle age, implying that they are part of a continuous aging process (Carrier et al., 1997, 2001, 2002). Age-related changes in sleep have also been observed in rodents. Consistent with human studies, older rats (20-24 mo) show a decline in SWS intensity and changes in the circadian organization of sleep (Stone, 1989; Mendelson & Bergmann, 2000; Shiromani et al., 2000). The mechanisms underlying age-related changes in sleep are poorly understood, but changes in endocrine status with aging may play a role.

1.1.3 Sex Differences in Sleep Patterns

Women of all ages are more prone to the detrimental effects of sleep loss than men (Hurst, 2008). Fluctuating levels of the major ovarian hormones, estrogen (E) and progesterone (P), during the menstrual cycle (Figure 3), pregnancy, and menopause have been associated with sleep disturbances and changes in EEG activity during sleep (Manber & Armitage, 1999; Moline et al., 2004; Dzaja et al, 2005; Baker & Driver,

2007). Sleep patterns also change in response to hormonal treatments, such as the use of oral contraceptives in young women (Baker et al., 2001; Burdick et al., 2002) and hormone replacement therapy (HRT) in post-menopausal women (Polo-Kantola et al., 1998; Antonijevic et al., 2000; Hachul et al., 2008). There is some debate in this field regarding the effects of HRT on sleep; some studies show evidence that hormone replacement therapy (E or E+P) can improve sleep quality in post-menopausal women under baseline conditions (Thomson & Oswald, 1977; Antonijevic et al., 2000; Polo-Kantola et al., 1998; Montplaisir et al., 2001; Hachul et al., 2008; Schussler et al., 2008), while other studies fail to find an effect of HRT on sleep quantity or quality (Polo-Kantola, 2011). Furthermore, it is unclear whether HRT can facilitate sleep recovery after sleep deprivation (Kalleinen et al., 2006). Therefore, it is important to assess the impact of HRT on both spontaneous sleep and recovery sleep following sleep deprivation.

1.2 Menopause

1.2.1 Menopausal Symptoms and Hormone Replacement Therapy

Menopause is the process during which a woman's ovaries stop producing mature ova and releasing E and P, and is accompanied by a gradual cessation of her menses. Many of the symptoms associated with menopause are due to the declining levels of ovarian hormones, including hot flashes, night sweats, which are commonly referred to as climacteric symptoms, as well as sleep problems, mood swings, irritability, irregular heartbeat, decreased libido and decreased responsiveness to sexual stimulation (Schumacher et al., 2007; Pluchino et al., 2011). HRT is the primary treatment option for

menopausal symptoms, particularly for climacteric symptoms (hot flashes and night-sweats).

HRT attempts to compensate for the loss of ovarian hormones by providing low doses of E, either alone or in combination with P. Synthetic Ps, as opposed to natural P, are often prescribed for HRT; synthetic Ps generally have more limited effects than natural P, as they do not form active metabolites (Schumacher et al., 2007). The safety of HRT has recently been called into question due to several large-scale clinical trials, which found increased adverse events in women receiving HRT in contrast to the placebo group. In particular, HRT administration was associated with increased risk of certain cancers, including ovarian and breast cancer, and cardiovascular problems such as coronary heart disease, stroke and venous thromboemboli (Hulley et al., 1998; Viscoli et al., 2001; Roussouw et al., 2002). However, subsequent analyses of these data revealed that these risks were found mostly in women initiating HRT many years after the onset of menopause, and that HRT was safe in women without co-morbid disorders when begun in the peri-menopausal period (NAMS, 2010). In fact, recent studies suggest that HRT may protect against cognitive decline and the development of Type 2 diabetes, and may also facilitate recovery after ischemic stroke (Roussouw et al., 2002; NAMS, 2010). The current recommendations are to prescribe the lowest effective dose of E in women, and to use caution when prescribing HRT to women with a history of, or with a high risk of developing, cancer or cardiovascular disease. E+P treatment is recommended for women with intact uteri, as P has protective effects against endometrial cancer (Schumacher et al., 2007; NAMS, 2010).

1.2.2 Estrogen and Progesterone in the Central Nervous System

E has many actions throughout the body, including the central nervous system (CNS). E acts through both slow signalling pathways mediated by nuclear receptors, and fast signalling pathways mediated by membrane-bound receptors (Björnström & Sjöberg, 2005). There are two main receptor sub-types, ERα and ERβ, each of which mediates both the slow- and fast-signalling pathways (Etgen & Garcia-Segura, 2009). The slow signalling pathways primarily contribute to the modulation of gene transcription; many genes contain estrogen response elements (Nilsson et al., 2001). The fast signalling pathways contribute to excitatory post-synaptic potentials (EPSPs), long-term potentiation (LTP) and intracellular signalling cascades (Gu & Moss, 1996, 1998; (Foy et al., 1999). ERα and ERβ are differentially distributed throughout the CNS; a summary of their distribution patterns, as they relate to brain areas associated with sleep-wake control, can be seen in Tables 1 and 2.

E directly modulates the activity of several NT systems, through both pre- and post-synaptic mechanisms, generally increasing neuronal excitability. These NTs include ACh, DA, NA, 5-HT, Glu and GABA. ERs have been found co-localized with neurons expressing these NTs. E modulates cholinergic transmission by increasing the activity and production of choline acetyltransferase (the rate limiting enzyme in ACh production), increasing stimulated release of ACh, and by enhancing the production and response of muscarinic ACh receptors (Etgen, 2002). E also has a facilitatory effect on DA transmission by attenuating D2-receptor (autoreceptor) inhibition of DA release and DA transporter activity, enhancing D1-receptor mediated EPSPs, reducing D2-mediated inhibitory post-synaptic potentials (IPSPs), as well as improving DA neuronal survival

with aging (Etgen & Garcia-Segura, 2009). Pre-synaptic NA release and post-synaptic responses to NA are also enhanced by E, particularly in the thalamus and preoptic areas; the actions of E in NA neurons are further facilitated by P (Etgen & Garcia-Segura, 2009). Similarly, E promotes 5-HT release, through actions on 5-HT transporters and autoreceptors, and post-synaptic responses, through regulation of 5-HT receptor expression and G-protein coupling; these actions are facilitated by P. Glu transmission is also enhanced by E, including presynaptic Glu release and regulation of ionotropic (NMDA and non-NMDA receptors) and metabotropic Glu receptor expression and function (Etgen & Garcia-Segura, 2009). E's effects on GABA neurotransmission vary depending on the brain area. In the hippocampus, E decreases the probability of GABA release (Rudick & Woolley, 2001); however, in the preoptic area (POA) E augments GABA release and this response is potentiated by P (Etgan, 2002). In addition, E causes increases in the density and affinity of $GABA_A$ (ionotropic) receptors in the preoptic area (POA) and hypothalamus (Etgen, 2002), and reduces $GABA_B$ (metabotropic) receptor density, G-protein coupling and binding affinity in the ventral tegmental area (VTA) and entorhinal cortex (Etgen & Garcia-Segura, 2009). E also regulates downstream effectors of G-protein signalling, such as cAMP, cGMP, adenylyl cyclase, inositol triphosphate, phospholipase C, calcium, nNOS and CREB; these effects further influence NT effects on their postsynaptic targets (Etgan & Garcia-Segura, 2009). Thus, E can have a variety of effect in various brain regions and influence a number of NT systems.

ERs are expressed throughout the CNS including brain regions involved in sleep and wake regulation. Specifically, ERs have been observed in significant percentages of cholinergic neurons in the basal forebrain (BF), DA neurons in the ventral periaqueductal

gray (vPAG), NE neurons in the locus coeruleus (LC), 5-HT neurons in the dorsal raphe nucleus (DR), Glu neurons in sublaterodorsal region (SLD), parabrachial (PB) and precoeruleus nuclei (PC), and GABAergic neurons in the preoptic area (POA), periaquedctal gray (PAG), lateral pontine tegmentum (LPT) and sublaterodorsal region (SLD)(Shughrue et al., 1997). E also activates SCN neurons and increases the frequency of EPSPs (Fatehi & Fatehi-Hassanabad, 2008). The ER expression in sleep/wake-regulatory areas is summarized in Tables 1 and 2

P signalling also occurs through both fast signalling pathways mediated by membrane bound receptors (mPR), and slow signalling pathways mediated by nuclear receptors PR-A and PR-B (Brinton et al., 2008). PRs are expressed in many sleep/wake promoting nuclei, including the lateral hypothalamus (LH), LC, DR, and BF, as well as in many other brain regions (Kato et al., 1994). Furthermore, the active metabolite of P, allopregnanolone, is a potent modulator of $GABA_A$ which enhances inhibitory neurotransmission mediated by $GABA_A$ receptors (Schumacher et al., 2007). P has neuroprotective effects mediated by MAPK signalling. P also upregulates the expression of anti-apoptotic proteins such as Bcl-2 and downregulates the expression of pro-apoptotic proteins, including Bax, Bad and caspase-3 (Schumacher et al., 2007).

The circulating levels of E can modulate the densities of both ERs and PRs. Treatment with E increases the expression of some PRs, particularly those in the hypothalamus and POA, but has no effect on others, including most of the PRs expressed in the cortex, hippocampus, amygdala and cerebellum (Mani & O'Malley, 2009). E's effects on ER distributions are not clear. Some studies show that E's effects on ER expression depend upon the timing of the treatment. When a low dose treatment is given

immediately following ovariectomy, ERα levels are up-regulated; however, when treatment is delayed by a few days ERα expression is down-regulated (Bohacek & Daniel, 2009). Various studies have shown a downregulation of ERs with E treatment, while others show no change in ER expression with E treatment. The discrepancy between these studies may be due to differences in technique; in particular, studies showing ER downregulation with treatment used *in situ* hybridization, whereas those showing no changes in ER used immunohistochemistry. Therefore, these differences may be due to differences between mRNA versus protein, and that there may be compensatory changes in posttranscription or translation in response to reduced mRNA levels following treatment (Chakraborty & Gore, 2004; Etgen & Garcia-Segura, 2009).

The expression and properties of ovarian hormone receptors can change with aging. Some studies have found region-specific changes in ERα expression and affinity with aging: aged rats show decreased E binding affinity to ERα, as well as decreased ERα expression in the hypothalamus, POA (particularly medial POA), ventromedial nucleus of the hypothalamus and pituitary when compared to young rats (Rubin et al., 1986; Brown et al., 1990). Age-related changes in ERβ expression also occur in rats and they are region-specific: downregulation of ERβ has been seen in the supraoptic nucleus, but no changes in expression were observed in the POA, MnPO or paraventricular nuclei (Chakraborty & Gore, 2004). Region-specific changes in PR expression associated with aging are also observed in E-treated rats: decreased P binding to PRs has been seen in the POA and medial basal hypothalamus, but not in the amygdala or pituitary (Chakraborty & Gore, 2004). Thus, it is clear that there are changes in ER and PR expression during aging that could modify the effect of E or E+P treatment on the CNS.

1.2.3 Menopause and Sleep

Many human studies provide evidence that there are increased sleep problems during the perimenopausal period; however, whether sleep problems are a primary symptom of menopause or whether they are secondary to climacteric symptoms is unclear. Sleep studies using self-report measures of sleep quality and quantity show a dramatic increase in sleep problems with aging and show that women with climacteric symptoms report poorer sleep quality and quantity (Polo-Kantola, 2011). Studies using objective sleep measures, such as actigraphy and polysomnography, provide conflicting evidence. Some studies report decreased sleep quality and/or quantity with aging that is independent of climacteric symptoms and that nocturnal awakenings preceded any climacteric symptoms observed (Freedman & Roehrs, 2007). Other studies report a relationship between sleep problems and hot-flashes, and that women with hot flashes have longer REMS latency and increased nocturnal arousals than women without hot flashes (Erlik et al., 1981; Shaver et al., 1991).

The efficacy of HRT to improve sleep quality has also been a topic of debate, as evidence from human studies is conflicting. Some researchers have found improved sleep quality with HRT, either E alone or E+P, while others have not found any improvement (Polo-Kantola, 2011). Furthermore, amongst researchers who support the view that HRT ameliorates sleep problems, there is debate whether this is due to direct effects of E or E+P on sleep or whether it is secondary to a reduction in climacteric symptoms (Nowakowski et al., 2009). Thus, it is important to determine what effects HRT has on spontaneous sleep patterns and on recovery sleep following sleep deprivation to help resolve conflicting clinical data.

Rodents have frequently been used as models to examine the effects of E and P on sleep. Rodent models of menopause have the advantage that sleep assessments are not complicated by climacteric symptoms, as they are not present in rats (Pawlyk et al., 2008a, 2008b). Recent studies from our laboratory demonstrated that estradiol and progesterone replacements have different effects on spontaneous sleep patterns than on recovery sleep following sleep deprivation in ovariectomized (OVX) young adult rats (Deurveilher et al., 2009, 2011). At baseline, E, either alone or combined with P, increased wake and decreased NREMS and/or REMS amounts. Poorer NREMS maintenance and consolidation were associated with E or E+P treatment, with a decrease in episode duration and an increase in number of episodes. Initiation of REMS at baseline was also impaired and the number of REMS episodes during the dark phase was lower in E or E+P treated animals. There was little or no difference between treatment groups in EEG delta or theta power during NREMS or REMS at baseline (Deurveilher et al., 2009, 2011). A summary of these findings is available in Table 3.

Deurveilher et al. (2009, 2011) also showed that E or E+P treatment altered the ability to recover lost sleep after acute (6 h) total sleep deprivation (SD) in OVX rats, enhancing REMS rebound and decreasing NREMS delta power. During the recovery period animals treated with E or E+P displayed longer mean durations of NREMS and REMS episodes, with fewer brief awakenings when compared to baseline sleep, unlike animals treated with an oil vehicle. This indicates improved sleep consolidation during recovery with E or E+P treatment. Nevertheless, the total amount of NREM sleep rebound did not change and there was a reduction in NREM EEG delta power in hormonally treated rats (Table 3) (Deurveilher et al., 2009, 2011).

Other studies have also found that estradiol treatment in ovariectomized (OVX) rats reduces sleep, especially REM sleep, under baseline conditions (Colvin et al., 1969; Branchey et al., 1971; Pawlyk et al., 2008a, 2008b). However, the effects of female sex hormones on recovery sleep following sleep deprivation may differ from those on baseline sleep.

Hormonal changes across the estrous cycle are also associated with changes in sleep patterns (Colvin et al., 1968; Yamaoka, 1978; Kleinlogel, 1983; Zhang et al., 1995; Kalleinen et al., 2006; Hadjimarkou et al., 2008). Despite baseline differences in sleep across the estrous cycle, intact rats did not show estrous cycle-related differences in amounts of NREM sleep, REM sleep, or NREM sleep delta power during recovery after 6 h of sleep deprivation (Schwierin et al., 1998). When rats are selectively deprived of REMS, but not NREMS, the estrous stage has been reported to affect the pattern of recovery sleep (Anderson et al., 2008).

These studies identify several differences between women and female rats in the effects of female sex hormones on sleep. For example, E decreased sleep duration in young OVX rats, whereas it increased sleep amounts in post-menopausal women, at least in some studies. A possible explanation for such discrepancy is the difference in life stages between subjects in these studies: loss of ovarian function and/or hormone replacement might have different effects in individuals of different ages due to changes in hormone receptor distribution and other age-related changes. Thus, using older, rather than young, female rodents might be more appropriate for modeling the impact of hormone loss and replacement on sleep in peri- and postmenopausal women. Furthermore, while baseline sleep patterns have been studied, the homeostatic response to

sleep deprivation and its modulation by ovarian hormones has not been well characterized in the context of aging.

1.3 Study Objectives

Hypothesis:

E or E+P treatment will impact baseline sleep architecture differently than recovery sleep after sleep deprivation in OVX middle-aged rats in a manner similar, but not identical, to what has been observed in OVX young adult rats.

Specific Aims:

1. To study whether E treatment, either alone or in combination with P, will increase the amount of wakefulness during baseline recordings at the expense of NREMS and decrease REMS in middle-aged OVX female rats.
2. To observe whether the effect of E and E+P will occur primarily in the dark or light phase during baseline recordings in middle-aged OVX female rats.
3. To investigate whether E treatment, either alone or in combination with P, will improve NREMS consolidation and REMS initiation during recovery after sleep deprivation compared to the corresponding baseline period in middle-aged OVX female rats.

4. To study the effects of E treatment, either alone or in combination with P, on EEG power spectra during baseline and recovery recordings in middle-aged OVX female rats.
5. To determine whether the general trends observed with E and E+P treatment will be similar to previously published data using young female OVX rats (Deurveilher et al., 2009, 2011), and what differences, related to age and other factors, are observed between young and middle-aged female rats.

CHAPTER 2 - EFFECTS OF FEMALE SEX HORMONES ON SLEEP AND RECOVERY FROM SLEEP DEPRIVATION IN MIDDLE-AGED OVARIECTOMIZED FEMALE RATS

2.1 INTRODUCTION

Age and ovarian hormone levels interact in a complex manner to influence sleep (Dzaja et al., 2005; Paul et al., 2008). Menopause can affect sleep differently depending on the woman's age at menopause onset. HRT may also have different effects on sleep when initiated at different ages or at different time points relative to menopause (Shahar et al., 2003; Haschul et al., 2008; Shuster et al., 2009). Furthermore, the interpretation of the effects of HRT on sleep in menopausal women is confounded by the presence of hot flashes that often occur in these women and themselves can cause sleep disruption (Pawlyk et al., 2008a, 2008b).

To address these issues we used middle-aged OVX female rats, with or without E or E+P replacement, to study the effects of the female sex hormones on spontaneous sleep patterns and on recovery sleep following acute SD. We used the well-established procedure for OVX combined with hormone replacement used in previous studies (Becker et al., 2005; Deurveilher et al., 2008, 2009, 2011; Mashoodh et al., 2008). Ovariectomy markedly reduces circulating E and P levels, and provides an appropriate experimental baseline condition to study the effects of each hormone. Hormone replacement is achieved by the subcutaneous implantation of hormone-filled capsules. These implants can provide stable levels of hormones for several weeks (Adams et al., 2006), which allow baseline sleep (24 h), sleep restriction (6 h), and subsequent recovery

sleep (24 h) to occur against a stable hormonal background, similar to what is achieved with HRT. This condition cannot be attained in intact female rats, in which hormonal levels fluctuate across the 4 to 5 day estrous cycle (Figure 3).

2.2 Materials and Methods

2.2.1 Animals

Middle-aged, retired breeder female Wistar rats (Harlan Laboratories, Indianapolis, IN, USA), 9-11 months old and 296-512g at the time of surgery, were used. All animals were housed under a 12/12 h light/dark cycle (lights on [Zeitgeber Time (ZT) 0] at 07:00) at 23 ± 1ºC ambient temperature. Rat chow and water were available ad libitum. The rats were randomly assigned to one of three treatment groups: Oil (n=5), E (n=5), or E+P (n=5). Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

2.2.2 Surgery

Middle-aged rats underwent surgery following a minimum of 14 days after arrival at the animal care facility to allow them to recover after transport. If the supplier indicated that there was the possibility of pregnancy, the rats were monitored for a minimum of 21 days (length of gestation in rats) prior to surgery to ensure that the rats were not pregnant at the time of surgery. Rats who delivered a litter during this period were allowed a minimum of 14 day post-partum recovery prior to surgery. Female rats

were OVX bilaterally under anesthesia. During the initial phase of the study, which accounted for one animal in each treatment group, surgeries were performed using a ketamine based anesthetic (72 mg/kg ketamine, 3.8 mg/kg xylazine, and 0.7 mg/kg acepromazine, i.p). However, as the mortality of middle-aged animals proved high with ketamine based anaesthesia, subsequent surgeries were conducted using inhaled isoflurane gas mixed with oxygen (4% induction; ≤ 2% maintenance; 1 L/min flow rate). An incision through the skin and muscle layers was made at the midline of the lower abdomen. The uterine horns were withdrawn from the abdominal cavity, the oviducts were clamped with forceps and tied with dissolvable sutures, and the ovaries were removed. The uterine horns were replaced inside the abdomen and the muscle layers were sutured.

Silastic capsules (1.6 mm inner diameter, 3.2 mm outer diameter; 45 mm in length for oil and estradiol, and 55 mm in length for progesterone (Dow Corning Corporation, Midland, MI, USA) were inserted subcutaneously, lateral to the incision, in each animal. All rats received one capsule, except for rats treated with both hormones (E+P), which received two. Capsules were filled with the following: sesame oil (Catalog No. S3547, Sigma-Aldrich, St Louis, MO); 17β-estradiol [most common natural subtype of E](65 µg; Catalog No. E8875, Sigma-Aldrich) in sesame oil; crystalline progesterone (66 mg; Catalog No. P0130, Sigma-Aldrich) group.

The dose of E given was reported to cause low plasma levels of E, characteristic of diestrus, 8 days after implantation in middle-aged rats (Dubal et al., 1998; Dubal & Wise, 2001). The 66 mg dose of P was calculated to give low blood levels of P comparable to diestrus levels based on doses from previous studies (Mannino et al.,

2005). See Table 4 for a summary of published dose-serum relationships. After capsule implantation, the skin incision was closed with surgical skin staples.

Subsequently, all animals were placed in a stereotaxic apparatus and implanted with 2 miniature stainless steel screws for EEG recording, one over the frontal cortex (1 mm rostral to bregma and 2 mm right of the midline) and the other over the occipital cortex (6 mm caudal to bregma and 2 mm left of the midline). A third screw was placed over the cerebellum (3 mm caudal to lambda and 2 mm right of the midline) to serve as a ground electrode. The screws were embedded into the skull to touch the surface of the dura matter. A pair of fluorocarbon-coated stainless steel wires with a 2–3 mm exposure was inserted into the nuccal muscles of the neck to record the electromyogram (EMG). All electrodes were connected to a small plastic connector (Plastics One Inc., Roanoke, VA), which was affixed to the skull with dental acrylic. Following surgery, animals were injected with an analgesic (Ketoprofen, 5 mg/kg) and an antibiotic (Enrofloxacin, 2.5 mg/kg) subcutaneously and monitored for recovery from anesthesia before being returned to the animal colony. Post-operatively the animals were singly housed. Skin staples were removed one week after surgery.

2.2.3 Experimental Design and Data Acquisition

Nine or 10 days after surgery, each rat was transferred to a clear Plexiglas cage ($40 \times 30 \times 40$ cm^3) placed inside an individual recording chamber that was equipped with a fan and an incandescent light controlled by a timer to maintain the same 12/12 h light/dark cycle as in the colony room. Food and water were available to the rats ad libitum while housed in the recording chamber. On the following day, rats were

connected to a flexible cable attached to a rotating commutator (Plastics One Inc.) and remained connected for a habituation period of 3–4 days before polygraphic recording started. The experimental procedures are illustrated in Figure 4.

EEG/EMG baseline recording began in the middle of the light phase, at 1 pm (ZT6), for a 24-h period, and was followed by 6 h of SD over the second half of the light phase (ZT6-12). Sleep deprivation was induced by introducing novel objects (plastic toys of different shapes and sizes) into their cages, gently moving their bedding, tapping on the side of their cage, and, when necessary, slowly moving their litter tray. These interventions were employed when the rats showed behavioural signs of sleepiness (i.e., when they assumed a sleep posture) or when slow waves were evident in the EEG. After sleep deprivation, EEG/EMG recording continued for 24 h, starting at the beginning of the dark-phase (ZT 12), to assess recovery sleep.

EEG and EMG signals were amplified and band pass-filtered (EEG: 0.3–100 Hz; EMG: 10–100 Hz; Grass Telefactor, West Warwick, RI). Signals were digitized at 256 Hz and stored on a computer for off-line analysis (SleepSign, Kissei Comtec America, Irvine, CA, USA).

2.2.4 Sleep-Wake Scoring and Analysis

Behavioural states were automatically scored in consecutive 10-sec epochs with each epoch identified as wake (low-voltage, fast EEG activity; moderate to high EMG activity), NREMS (high-voltage EEG activity, predominantly delta waves [0.5–4 Hz]; low amplitude EMG), or REMS (low-voltage EEG activity, predominantly theta waves

[4.5–8 Hz]; very low EMG activity with occasional muscle twitches). The automatic scoring was visually inspected and corrected (< 10% disagreement).

EEG power spectra during wake, NREMS and REMS were analyzed in 0.5-Hz bins using a fast Fourier transform (FFT; Hanning window) in 2 sec bins. Power values were averaged over a 10 sec epoch, and the mean value for 2 h or 12 h intervals was normalized to the total power for the corresponding time interval in each animal. Epochs with EEG artifacts (8.7% of total sleep epochs) were excluded from the FFT analysis.

2.2.5 Tissue Collection and Radioimmunoassay

At the end of the 48 h recording period, the rats were anaesthetized to a surgical plane using inhaled isoflurane and decapitated. Blood samples were collected in heparinized vials and then centrifuged at 3000 rpm for 10 min. Plasma was collected and kept frozen at −80°C until radioimmunoassay. Plasma concentrations of estradiol and progesterone were determined using commercial kits (Estradiol: Catalog No. DSL-4800, Beckman-Coulter, Fullerton, CA, USA; Progesterone: TKPG1; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The detection limit of the assays was 5 pg/mL for estradiol and 0.1 ng/mL for progesterone. The intra-assay coefficient of variation was 13.1% for estradiol and 6.5% for progesterone. All assays were conducted in a single session. Uteri were harvested immediately after blood collection and weighed.

2.2.6 Statistical Analyses

Statistical analyses were performed with Statview 5.0 (SAS Institute Inc., Cary, NC, USA) and SPSS 14.0 (SPSS Inc, Chicago, IL, USA). Sleep/wake parameters were

analyzed using a one-way repeated measures ANOVA to compare multiple time points. Serum progesterone levels and uterus weights were analyzed using one-way ANOVAs. Post hoc multiple comparison analyses (LSD, Game-Howell or paired t-tests) were used to further analyze significant main effects and interactions. For serum estradiol levels, nonparametric Kruskal-Wallis tests were conducted, and nonparametric Dunn post hoc tests were used to determine group differences. Probabilities of less than 0.05 were considered statistically significant.

2.3 RESULTS

2.3.1 Plasma E and P Levels, Uterus Weights, and Body Weights

Hormone treatment efficacy was confirmed by radioimmunoassays of plasma estradiol and progesterone, and bioassays of uterus and body weights (Table 5). A non-significant increase in estradiol level was observed in the E and E+P treated groups compared to the Oil treated group. The lack of significance was due to outliers in all three treatment groups with either non-detectable or extremely elevated values. The reasons for these outliers is not clear, but might be related to some thawing of the samples that occurred during transport.

Plasma progesterone levels were higher in the E+P group than in the Oil or E groups ($F_{2,12} = 11.63$, $P < 0.05$; E+P > Oil and E, both $P < 0.05$). The E and E+P treated animals showed increased uterus weight compared to Oil treated animals ($F_{2,12} = 29.50$, $P < 0.05$; E and E+P > Oil, both $P < 0.05$). The differences in uterus weight remained significant when they were normalized to body weight at the time of sacrifice ($F_{2,12} = 38.79$, $P < 0.05$; E and E+P > Oil, both $P<0.05$).

Unlike previous studies (Deurveilher et al., 2009,2011) using young female rats, we observed a large reduction in absolute body weight (mean of 47.8 to 52.4 g; Table 5) from the time of surgery to sacrifice (~3 weeks) in older females of all treatment groups. However, there was no effect of hormonal treatment on body weight change between surgery and sacrifice, nor was there any correlation between body weight change and any sleep/wake parameter in the baseline recording period that are described below.

2.3.2 Baseline Sleep-Wake States: Amounts, Frequency and Duration

2.3.2.1 Wakefulness

During the 24 h baseline period (Figure 5A) both E and E+P treated animals showed elevated amounts of wake, compared to Oil treated animals ($F_{2,12} = 4.06$, $P < 0.05$; E (+119 min) and E+P (+100 min) > Oil, both $P < 0.05$). This effect was more prominent in the dark phase (Figure 5C), with E treated animals showing more wake than the Oil group ($F_{2,12} = 4.32$, $P < 0.05$; E > Oil, $P < 0.05$). Treatment did not affect the duration or number of wake episodes during baseline recording (Figures 6 and 7; Tables 6 and 7). There were also no differences between groups in the ratio of amount of time spent awake during the light versus the dark phase (Figure 8).

2.3.2.2 NREM Sleep

During the 24 h baseline period (Figure 5A) both E and E+P treated animals showed decreased amounts of NREMS, compared to Oil treated animals ($F_{2,12} = 4.27$, $P < 0.05$; E (-99 min) and E+P (-95 min) > Oil, both $P < 0.05$). This effect was more prominent in the dark phase (Figure 5C), with E and E+P treated animals showing more

wake than the Oil group ($F_{2,12} = 4.92$, $P < 0.05$; E and E+P > Oil, $P < 0.05$). There was no significant effect of treatment on the duration or number of NREMS episodes over the 24h (Figures 6A and 7A; Tables 6 and 7) or the light phase periods (Figures 6B and 7B; Figure 12; Tables 6 and 7) of baseline recording. During the baseline dark phase, however, E and E+P treated animals showed significantly shorter NREMS episode duration ($F_{2,12} = 7.19$, $P < 0.05$; E and E+P > Oil, $P < 0.05$) (Figures 6C and 12A; Table 6), but no difference in the number of episodes of NREMS (Figures 7C and 12B; Table 7). There were no differences between groups in the light/dark ratio of amount of time spent in NREMS (Figure 8)

2.3.2.3 REM Sleep

During the 24 h baseline period (Figure 5A), light phase (Figure 5B) and dark phase (Figure 5C), there was no effect of treatment on the amount of REMS. There was also no significant effect of treatment on the duration of REMS episodes, although there was a trend for longer REMS episodes in the E and E+P treated animals, compared to the Oil group over the 24 h (Figures 6A; Table 6), during the light (Figures 6B; Figure 13A; Table 6), and dark phase periods (Figures 6C and 13B; Table 6) of baseline recording. In contrast, during the baseline dark phase, E treated animals showed significantly fewer REMS episodes compared to the Oil group ($F_{2,12} = 4.67$, $P < 0.05$; E > Oil, $P < 0.05$)(Figures 7C and 13B; Table 7), with a trend for fewer REMS episodes in the E+P treated animals compared to the Oil group. A trend for fewer REMS episodes in the E and E+P treated animals compared to the Oil group was also observed over the 24 h baseline period (Figure 7A; Table 7) and during the light phase (Figures 7B and 13B;

Table 7of baseline recording. There were no significant differences between groups in the light/dark ratio of amount of time spent in REMS; however, the E and E+P tended to show higher ratios (Figure 8).

2.3.3 Sleep Deprivation

Depriving the animals of sleep for 6 h by gentle handling, during the second half of the light phase, was successful in keeping them awake 98% of the time for all groups. The number of interventions required to keep the animals awake increased over the course of the SD period (Figure 9) indicating increasing sleep pressure over the course of SD. There was no effect of treatment on the number of interventions required over time.

2.3.4 Recovery Sleep after Sleep Deprivation

2.3.4.1 Wakefulness

All groups showed a decreased amount of wake during the 24 h recovery period compared to 24 h baseline ($F_{1,12} = 77.82$, $P < 0.05$) with no effect of treatment. The decrease in amount of wake was primarily observed during the first half (dark phase) of recovery (Figure 10A), with E treated animals showing more wake than the Oil group ($F_{1,12} = 102.76$, $P < 0.05$; E > Oil, $P < 0.05$). However, relative decrease from baseline during the same period was not different between groups (Figure 10D). In terms of time course, the first 2 h of the dark phase (Figure 14), which immediately followed the SD period, was associated with a particularly prominent decrease in amount of wake compared to baseline in all 3 groups ($Oil_{rec} < Oil_{bsl}$, $F_{1,4} = 27.15$, $P < 0.05$; $E_{rec} < E_{bsl}$, $F_{1,4} = 15.62$, $P < 0.05$; $E+P_{rec} < E+P_{bsl}$, $F_{1,4} = 21.40$, $P < 0.05$). The reduction in amount of wake

continued for the rest of the dark phase, although the size of the effect tended to diminish over time; the amount of wake returned to baseline levels for all groups during the second half (light phase) of recovery (Figures 11A and 14).

The mean duration of wake episodes was significantly shorter during recovery than baseline ($F_{1,12} = 11.59$, $P < 0.05$; $Oil_{bsl} > Oil_{rec}$, $P < 0.05$) (Table 6); this decrease was primarily observed during the first half (dark phase) of recovery ($F_{1,12} = 18.04$, $P < 0.05$; $Oil_{rec} < Oil_{bsl}$ & $E+P_{rec} < E+P_{bsl}$, both $P < 0.05$, trend for $E_{rec} < E_{bsl}$, n.s.). The duration of wake episodes returned to baseline values for the second half (light-phase) of recovery. The mean number of wake episodes (Table 7) was higher during recovery than baseline in all 3 groups selectively in the first 12 h (dark phase) of recovery ($F_{1,12} = 9.88$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$ & $E+P_{rec} > E+P_{bsl}$, both $P < 0.05$, trend for $E_{rec} > E_{bsl}$, n.s.). The duration of wake episodes returned to baseline values for the second half (light-phase) of recovery.

2.3.4.2 NREM Sleep

All groups showed an increased amount of NREMS during 24 h recovery compared to 24 h baseline ($F_{1,12} = 48.74$, $P < 0.05$) with no differences between treatment groups. This increase was observed mainly during the first half (dark phase) of recovery (Figure 10B), with E treated animals showing less NREMS than the Oil group ($F_{1,12} = 61.89$, $P < 0.05$; $E > Oil$, $P < 0.05$). However, relative increase from baseline in NREMS amount during the same 12 h period did not differ between groups (Figure 10D). The first 2 h of the dark phase (Figure 15) immediately after SD was associated with a particularly prominent increase in NREMS amount compared to baseline in all 3 groups

($Oil_{rec} > Oil_{bsl}$, $F_{1,4} = 31.35$, $P < 0.05$; $E_{rec} > E_{bsl}$, $F_{1,4} = 21.50$, $P < 0.05$; $E+P_{rec} > E+P_{bsl}$, $F_{1,4} = 25.83$, $P < 0.05$). The elevation in NREMS amount continued for the rest of the dark phase, although the size of the effect diminished gradually over time, and more rapidly in the E group than in the Oil or E+P group (Figure 15). The amount of NREMS returned to baseline levels for all groups during the second half (light phase) of recovery (Figures 11B and15).

The mean duration of NREMS episodes was not different over the 24 h recovery period versus the 24 h baseline (Table 6). However, an increase in NREMS episode duration (Figure 12A; Table 6) was observed during the dark phase of recovery compared to baseline for all 3 groups ($F_{1,12} = 41.54$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$, $E_{rec} > E_{bsl}$, & $E+P_{rec} > E+P_{bsl}$, all $P < 0.05$). The duration of NREMS episodes returned to baseline values for the second half (light-phase) of recovery (Figure 12A; Table 6), except for the Oil group, which showed a negative rebound ($F_{1,12} = 4.38$, $P < 0.05$; $Oil_{rec} < Oil_{bsl}$, $P < 0.05$). There was no difference between groups in the percentage change from baseline in NREMS episode duration during either the dark or light phases. The mean number of NREMS episodes (Figure 12B; Table 7) was higher during the 24 h recovery period than during baseline in all 3 groups ($F_{1,12} = 4.23$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$, $P < 0.05$, trend for $E_{rec} > E_{bsl}$ & $E+P_{rec} > E+P_{bsl}$, n.s.), and this increase was particularly prominent during the first half (dark phase) of recovery ($F_{1,12} = 61.89$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$ & $E+P_{rec} > E+P_{bsl}$, both $P < 0.05$, trend for $E_{rec} < E_{bsl}$, n.s.). The number of wake episodes returned to baseline values for the second half (light-phase) of recovery (Figure 12A).

2.3.4.3 REM Sleep

All groups showed an increased amount of REMS during 24 h recovery compared to 24 h baseline ($F_{1,12} = 75.95$, $P < 0.05$) with no treatment effect. This increase was primarily observed during the first half (dark phase) of recovery (Figure 10C)($F_{1,12} = 201.42$, $P < 0.05$). The relative increase from baseline tended to be higher in the E group than in the Oil or E+P group (Figure 10D). The first 2h of the dark phase (Figure 16) showed an increase in REMS amount during recovery compared to baseline in Oil and E treated groups ($Oil_{rec} < Oil_{bsl}$, $F_{1,4} = 5.98$, $P < 0.05$; $E_{rec} < E_{bsl}$, $F_{1,4} = 3.66$, $P < 0.05$; trend for $E{+}P_{rec} < E{+}P_{bsl}$, n.s.). The elevation in REMS amount continued for the rest of the dark phase, unlike the observed rebound increase in NREMS which quickly diminished . The amount of REMS returned to baseline levels for all groups at the beginning of, and throughout the second half (light phase) of recovery in the E group (Figures 11C and 16); however, the Oil group showed a negative rebound around the mid-light phase (Figure 16A), with a trend of an overall decrease in REMS during the light phase (Figure 11C). In contrast, in the E+P group, REMS amount continued to be elevated during the second half (light phase) of recovery ($F_{1,12} = 5.98$, $P < 0.05$; $E{+}P_{rec} > E{+}P_{bsl}$, $P < 0.05$)(Figures 11C and 16).

The mean duration of REMS episodes was not different over the 24 h recovery period versus the 24 h baseline (Table 6). However, an increase in REMS episode duration (Figure 13A; Table 6) was observed during the dark phase of recovery compared to baseline in all groups, and this increase was significant for the Oil treated group ($F_{1,12} = 41.54$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$, all $P < 0.05$; trend for $E_{rec} > E_{bsl}$, & $E{+}P_{rec} > E{+}P_{bsl}$, n.s.), but there was no difference in the percentage change from baseline between any treatment groups in REMS episode duration during the dark phase of recovery. The

duration of REMS episodes returned to baseline values for the second half (light-phase) of recovery (Figure 13A; Table 6) for all groups, except for the E+P group, for which REMS episode duration continued to be elevated ($F_{1,12} = 4.11$, $P < 0.05$; $E+P_{rec} > E+P_{bsl}$, $P < 0.05$). The E+P group also showed an elevated percent change in REMS episode duration from baseline during the light phase of recovery, compared to the Oil group ($F_{2,12} = 4.03$, $P < 0.05$; E+P > Oil, $P < 0.05$). The mean number of REMS episodes (Figure 13B; Table 7) was higher during recovery than baseline in all 3 groups over the 24 h recovery ($F_{1,12} = 10.52$, $P < 0.05$; $E+P_{rec} > E+P_{bsl}$, $P < 0.05$, trend for $Oil_{rec} > Oil_{bsl}$ & $E_{rec} > E_{bsl}$, n.s.). This increase over a 24 period was due to a dramatic two-fold increase during the first half (dark phase) of recovery that was observed in all 3 groups ($F_{1,12} = 40.68$, $P < 0.05$; $Oil_{rec} > Oil_{bsl}$, $E_{rec} > E_{bsl}$, & $E+P_{rec} > E+P_{bsl}$, all $P < 0.05$). Nevertheless, E and E+P treated animals showed significantly fewer episodes of REMS than the Oil group during the dark phase of recovery ($F_{1,12} = 6.24$, $P < 0.05$; E & E+P < Oil, $P < 0.05$). The number of REMS episodes returned to baseline for the second half (light-phase) of recovery.

2.3.5 EEG Power Spectral Analyses

2.3.5.1 Baseline Power Spectra

Power spectral values were normalized to the total power for each respective sleep/wake state. As expected, the EEG power spectra during baseline NREMS showed a peak in the delta range (0.5-4 Hz), while REMS EEG power spectra showed a peak in the theta range (4.5-8 Hz). There were no differences between treatment groups in baseline power spectra for any frequency range, except for a small increase in gamma

power in the dark phase during wake episodes (Figure 17D) in E+P treated animals ($F_{2,12}$ = 4.28, $P < 0.05$; E+P > Oil & E, $P < 0.05$). The E group tended to show lower delta power and higher theta power during REMS at baseline regardless of time of day (Figure 17C,F).

2.3.5.2 Recovery Power Spectra

As expected, the NREMS EEG power spectra during recovery showed a peak in the delta range (0.5-4 Hz), while REMS EEG power spectra showed a peak in the theta range (4.5-8 Hz). There were no differences between treatment groups in recovery power spectra for any behavioural state, except for a small increase in gamma power ($F_{2,12}$ = 4.64, $P < 0.05$; E+P > Oil & E, $P < 0.05$) and a decrease in delta power in the dark phase during wake episodes (Figure 18D) in E+P treated animals ($F_{2,12}$ = 3.68, $P < 0.05$; E+P > Oil & E, $P < 0.05$). As was the case at baseline, E treated animals tended to show lower delta power and higher theta power during recovery REMS regardless of time of day (Figure 18C,F).

EEG delta power during NREMS is considered to be a measure of sleep intensity. The time course analysis of normalized delta power during NREMS shows elevated NREMS delta power during the first 4 hours of recovery, in the dark phase (Figure 19), for all treatment groups ($F_{2,12}$ = 21.64, $P < 0.05$). Rebound increases in NREMS delta power gradually decreased over the course of the dark phase of recovery, with the Oil group showing an earlier reduction in NREMS delta power, compared to baseline, than the E and E+P groups and returned to baseline values for the subsequent light phase.

2.4 Discussion

The present results indicate that treatment with estrogen, or estrogen combined with progesterone, over a 2-week period alters spontaneous sleep patterns and the sleep architecture during recovery after sleep deprivation in ovariectomized middle-aged female rats. During the baseline recording, animals treated with E or E+P displayed decreased amounts of NREMS, decreased duration of NREMS episodes, decreased number of REMS episodes, and increased amounts of wakefulness, compared to Oil treated rats. These changes were selective to the dark phase. During the first 12 h of recovery following sleep deprivation, which occurred in the dark phase, E treated, but not E+P treated, animals showed a smaller NREMS rebound with a less pronounced reduction in wakefulness, compared to Oil treated animals. E+P treated animals showed particularly prolonged REMS rebound compared to E and Oil treated animals, with increased REMS, due to longer REMS episodes, continuing into the second 12 h (light phase) of recovery. These results indicate that hormone treatments affect sleep patterns differently during baseline and recovery periods in ovariectomized middle-aged rats, possibly due to hormonal modulation of homeostatic and other regulatory mechanisms.

The E and E+P treatments raised serum estradiol levels to diestrus levels (7-20 pg/mL). The changes in uterus weights confirmed treatment efficacy in E and E+P treated rats, with an approximate 3-fold increase in uterus weight compared to Oil treated animals, consistent with previous studies (Ke, 1997; Gogos, 2004). Serum progesterone levels were also raised to diestrus levels (3-30 ng/mL) in E+P treated animals. Diestrus serum estrogen and progesterone levels were chosen as our target for dosing in order to

mimic current treatment recommendations for HRT in menopausal women (NAMS, 2010).

Unlike previous studies (Ke, 1997; Gogos, 2004; Deurveilher et al., 2009, 2011), body weights were not affected by the hormone treatments; all animals, in fact, showed weight loss, not weight gain, over the two weeks from the time of surgery to sacrifice. The reason for this difference is not clear; however, I offer two possible reasons. First, due to their age, these animals might have required a longer period of recovery following surgery than the young animals used in previous studies. This could have limited their mobility for a longer period and prolonged discomfort, which could have altered their eating habits longer post-operatively, thus, causing weight loss. Second, any weight gain might have been masked by faster loss of adipose tissues during postoperative recovery in older animals. This can occur because the amount of weight loss was proportional to initial body weight and most of these animal were overweight, often almost to the point of obesity, and adipose tissues are the first to be lost in case of metabolic challenge as occurs after surgery (Markowska & Savonenko, 2002). The post-operative weight loss would then have been more pronounced than would be observed in younger animals, which would not have as much adipose tissue to lose. Thus, the post-operative weight loss could have overshadowed any subsequent weight gain due to treatment.

We considered the possibility that negative energy balance as a result of weight loss caused or contributed to the observed changes in sleep/wake patterns. However, the amount of weight loss was similar between the three treatment groups. Furthermore, there was no correlation between the amount of weight loss and any of the sleep and wake measures analyzed for baseline recordings. These observations suggest that weight loss is

unlikely to be the primary reason for the observed effects of hormonal treatment on sleep/wake patterns.

2.4.1 Estradiol and Progesterone Replacement Reduces Baseline Sleep

During baseline recording of spontaneous sleep patterns, animals receiving E treatment, either alone or in combination with P, spent less time in NREMS, with concomitant increases in wakefulness, compared to Oil treated animals. This reduction in NREMS in E and E+P groups was mainly due to shorter NREMS episodes; there was no change in NREMS frequency. These perturbations in NREMS patterns were observed over the 24h period, but the effects were greater during the dark (active) phase. These findings suggest that E treatment compromises NREMS maintenance, particularly in the dark phase.

E treated animals showed fewer episodes of REMS during the dark phase than oil treated animals; a similar, but non-significant, effect was observed in E+P treated animals. Interestingly, there was no significant difference between groups in total REMS amount or in REMS episode duration, although there was a trend for longer REMS episode duration in the E and, to a lesser degree, E+P treated animals. These results imply that E treatment impairs REMS initiation, but that this effect is mitigated by simultaneous treatment with P. Furthermore, it appears that deficits in REMS initiation may be accompanied by a compensatory increase in REMS episode duration. Whether the disruptions in REMS initiation are due to direct hormonal effects, or whether they are a secondary effect of impaired NREMS maintenance is unclear. However, it should be noted that REMS initiation generally requires a lengthy preceding period of NREMS.

Interestingly, there were no effects of hormonal treatment on the EEG delta power (a measure of sleep intensity) during NREMS at baseline, indicating that although the NREMS amounts were reduced in the E and E+P treated animals, there were no compensatory increases in sleep intensity in these animals. There was, however, an increase in gamma power during wake in the dark (active) phase of baseline in the E+P treated animals, presumably reflecting an increased arousal level in these animals. All observed effects on baseline EEG occurred primarily during the dark phase of recording.

E and E+P treated animals showed more robust light/dark differences than Oil treated animals, in both sleep/wake patterns and EEG power spectra. This may be due to E activity in the SCN, the principal brain area involved in the circadian regulation of sleep. It is interesting that E treatment appears to affect the light/dark ratios of all aspects of NREM and REMS, including sleep amounts, intensity, and episode duration and frequency.

Our results indicate that E and E+P treatment affects spontaneous sleep patterns in middle-aged rats, but whether age plays a role in mediating these effects is not clear. A previous study in our laboratory by Deurveilher et al. (2009, 2011) examined the effects of ovarian hormones in young ovariectomized female rats on baseline and recovery sleep. These studies used a low dose of E, either alone (LE) or in combination with a low dose of P (LEP), to achieve diestrus serum levels of these hormones as in the present study, as well as a higher dose of estrogen (HE), which raised blood serum to proestrus levels, a low dose of P (LP), and oil vehicle controls (Oil). They observed increased amounts of wake and reduced NREMS episode durations in all E treated groups (LE, LEP, and HE) at baseline, and this effect was primarily observed during the dark phase. However, the

increase in wake occurred mainly at the expense of REMS amounts, an effect that was more robust in the LEP group than the HE or LE groups. Interestingly, they also observed a decrease in NREMS amount and fewer REMS episodes, but only for the HE and LEP groups, and not LE group. Similar to the current study, there were no changes in EEG power spectra during any sleep/wake state.

As the same experimental protocol was used in the current study and Deurveilher et al. (2009, 2011), it is possible to compare the results from control rats (ovariectomy with oil treatment) in the two studies to speculate on any possible effects of aging. Due to several differences between the animals used in each study, including reproductive experience, direct statistical comparisons would be invalid; therefore, we will discuss the trends and qualitative differences between the results of the young and middle-aged Oil treated rats. A summary of these comparisons are available in Table 9.

The absolute amounts of NREMS over the 24 h baseline period were higher in middle-aged animals. NREMS episodes had similar durations in both age groups, with a similar mean episode duration in both the light and dark phases. The increased amount of NREMS in the middle-aged was mainly due to an increased NREMS frequency during the light (rest) phase. As a result, middle-aged animals showed a greater light/dark ratio in NREMS than reported for young animals by Deurveilher et al. (2009, 2011). These results indicated that there was an overall increase in NREMS amount in middle-aged animals, compared to young animals; whether the differences between age groups is due to age or an unknown factor is unclear. However, it should be noted that current literature asserts that there is a reduction, rather than an elevation, in sleep amounts with aging (Polo-Kantola, 2011).

REMS patterns also differed between middle-aged and young Oil treated ovariectomized animals, but in a different pattern compared to NREMS, with middle-aged animals showing decreased amounts of REMS in both the light and dark phases. The reduced REMS amount in the dark (active) phase was due to shorter REMS episodes, with no changes in episode frequency. In the light phase, middle-aged animals showed a further reduction in REMS episode duration; however, since the episode frequency was also increased in the light phase, there was only a modest reduction in total REMS amount. This reduction in REMS amount and episode duration in middle-aged animals implies an alteration in REMS maintenance. This result agrees with current literature, which claims that the amount of sleep diminishes with aging (Polo-Kantola, 2011).

The general trends of increased wake, impaired NREMS maintenance and impaired REMS initiation, as well as increased light/dark ratio observed with E treatment in middle-aged female rats in the present study were similar to those reported for young female rats by Deurveilher et al. (2009, 2011). However, there were several interesting differences in the effects of hormonal treatment between the young and middle-aged animals used in these studies. First, the effects we observed with our E treatment group were most similar to the HE treatment group, not the LE group, in the Deurveilher et al. (2009, 2011) study, despite the similarity in estradiol serum levels (E = 14.7 pg/mL in the present study; LE = 13.4 pg/mL, and HE = 32.2 pg/mL in Deurveilher et al., 2009) in our E and their LE groups. This suggests that the potency of E may increase in middle-aged versus young animals. Second, there was no change in REMS amount in our E treated animal, because, while REMS frequency decreased, REMS episodes also lengthened. In

contrast, Deurveilher et al. (2009, 2011) reported reductions in REMS amounts, but no changes in episode duration. Whether these differences in the responses to E treatment are due to changes in sleep regulation with aging, or whether they are due to unknown confounding factor(s) associated with one or the other of the animal groups, cannot be determined at this time; however, further studies of these differences are warranted.

The effects of adding progesterone to E drastically differed between the two studies. In middle-aged animals (the current study), the E+P and E treatments generally had similar effects, and, where differences were found, the addition of P tended to reduce the effects of E (e.g. the number of REMS episodes). In contrast, in young rats (Deurveilher et al., 2009, 2011), the addition of P to E treatment amplified the observed effect of E; the LEP group showed more robust responses than the LE group, and even compared to the HE group (e.g. the number of brief awakenings). The apparent reversal of P effects in middle-aged, as compared to young, animals may be due to possible age-related changes in PR expression patterns or kinetics of PRs. It should also be noted that higher serum progesterone levels were present in our E+P group (8.4 ng/mL) than were reported by Deurveilher et al. (2009) in the LEP group (4.4 ng/mL). There may also be unknown factors that differed between the two studies, and these various possibilities are not mutually exclusive. A summary of the comparisons between middle-aged and young animals can be seen in Table 8.

Our results showing the effects of ovarian hormone on sleep/wake patterns and intensity are also consistent with many other published studies. Young intact female rats show decreased amounts of sleep on proestrus nights, immediately after peak levels of E occur (Fang & Fishbein, 1996; Schwierin et al., 1998; Hadjimarkou et al., 2008) and E

has also been reported to have an arousal-promoting effect in rodents (Pfaff et al., 2002). A study looking at the effects of 17β-estradiol in young mice also found decreased amounts of NREMS during the dark phase of baseline recordings (Paul et al., 2009). Our results are also consistent with a study looking at the effects of estradiol benzoate, a synthetic E with a longer half-life than 17β-estradiol, in ovariectomized rats, which found decreased NREMS, due to decreased NREMS episode duration, during the dark phase (Matsushima & Takeichi, 1990). In contrast, a recent study looking at the effect of 17α-ethinyl estradiol, another synthetic E with a longer half-life than 17β-estradiol, in ovariectomized young female rats found decreased duration, but not number, of episodes of REMS during the dark phase (Pawlyk et al., 2008a). This discrepancy could be due to a number of factors, including the differences in the age of the rats (middle-aged versus young), the strain of rat (Wistar versus Sprague-Dawley), the route of administration (s.c. implants versus i.v. injections), or the duration of treatment prior to sleep assessment (2 weeks versus 4 days). Another explanation is that different forms of E can have varying affinities for ERα and ERβ (Kuhl, 2005), and differ in their effects on their regulation of ER subtype expression (Osterlund et al., 1998; Patisaul et al., 1999; Jin et al., 2005).

The mechanisms underlying the effects of E and P on sleep/wake states are largely unknown. However, it is possible to speculate that E and P act on the sleep/wake system. It is possible that E and P could have direct actions on sleep/wake regulation as ERs and PRs have been found in various brain nuclei involved in sleep and wake, including the hypothalamus, BF, POA, DR and LC (Tables 1 and 2). Consistent with this hypothesis, sleep deprivation-induced c-Fos expression (a marker of neuronal activation)

in sleep/wake areas has been shown to be modulated by E treatment (Peterfi et al., 2004; Deurveilher et al., 2008; Hadjimarkou et al., 2008).

E and, to a lesser extent, P are also known to directly modulate neurotransmission, generally in a stimulatory manner resulting in increased neuronal excitability. E has been shown to increase evoked release of ACh, an important NT in the wake promoting nuclei in the pontine tegmentum (Etgen, 2002), which could result in increased amounts of wake. E has also been shown to augment the release of inhibitory GABA in the POA, a region involved in both NREM maintenance ($VLPO_c$) and REM initiation ($VLPO_e$), and this response is potentiated by P. Thus, it is possible that there is increased inhibition of the VLPO through GABAergic neurotransmission, suppressing this region's sleep promoting function (Etgen, 2002). E has also been proposed to increase wake through decreased expression of prostaglandin D synthase in the VLPO. Prostaglandin D synthase is an enzyme involved in the production of the sleep promoting factor prostaglandin D_2 (Mong et al., 2003; Hadjimarkou et al., 2008). Decreased production of adenosine A_{2a} receptors, which bind the sleep promoting factor adenosine, by E in the VLPO may also promote wake (Ribeiro et al., 2009).

P can also modulate neurotransmission via its active metabolites, including allopregnanolone, which is a potent modulator of $GABA_A$ receptors, and thus enhances GABA neurotransmission (Schumacher et al., 2007). Metabolites of P have been reported to have hypnogenic, anxiolytic and analgesic properties due to their interaction with $GABA_A$ receptors (Schumacher et al., 2007; Brinton et al., 2008; Caufriez et al., 2011). This could mediate the increased number of REMS episodes we observed in E+P compared to E treated groups, through inhibition of wake-promoting nuclei.

Interpretation of P's activity and effects is further complicated, as active metabolites of P can also modulate the activity of various other NT receptors, including nicotinic ACh receptors, glycine receptors, 5-HT receptors, NMDA receptors, AMPA receptors, and kainate receptors (Rupprecht & Holsboer, 1999).

The effects of E and E+P on the circadian regulation and the light/dark ratio may be mediated by effects of hormonal treatment in the SCN. The SCN houses the principal circadian clock, which can be entrained by ambient light, and ERs are present in this nucleus (Shughrue et al., 1997; Wilson et al., 2002; Vida et al., 2008). The SCN responds to E treatment with increased frequency of neuronal firing in vitro (Fatehi & Fatehi-Hassanabad, 2008), increases in light-induced expression of transcription factors (Abizaid et al., 2004), and shifts in the rhythm of clock-related gene expression in vivo (Nakamura et al., 2005). E modulation of gene transcription and neuronal firing in the SCN or photic entrainment may, therefore, be responsible for the more robust light/dark ratio and phase selectivity of effects of E and E+P treatment, which occurred primarily in the dark phase. It is also possible that E and E+P's effects on the light:dark variation of sleep/wake measures are secondary to other diurnally regulated processes, such as body temperature regulation.

2.4.2 Estradiol and Progesterone Replacement Promotes REMS Recovery after Sleep Deprivation

The SD procedure was effective in keeping all animals awake for at least 98% of the 6 h SD period. All animals required an increasing number of interventions over the course of SD, but there were no differences between groups, indicating a similar time

course of accumulation of sleep pressure across the groups. These data indicate that sleep deprivation was successful in increasing sleep propensity and that all animals reached similar levels of sleep pressure at the end of SD.

In response to 6 h of sleep deprivation, animals in all groups showed the typical rebound increases in NREMS and REMS amounts during the first half (dark phase) of recovery compared to baseline, although the increase in NREMS did not reach statistical significance in E treated animals due to high variability. E treated animals showed decreased absolute amounts of NREMS during recovery, compared to the Oil group. Overall, however, the percent change in NREMS and REMS amounts from baseline values did not differ significantly between groups.

All groups also showed an increase in both duration and number of NREMS episodes during the first 12 h (dark phase) of recovery, which did not differ significantly between groups. It is interesting that during recovery there were no differences between treatment groups, which is in direct contrast to baseline data, where E and E+P treated animals showed impaired NREMS maintenance. These data imply that increased sleep pressure promoted both initiation and maintenance of NREMS in E and E+P treated animals, during the recovery period compared to the baseline period. This NREMS promotion could be due to direct effects of the ovarian hormones at nuclei involved in the homeostatic response, such as the VLPO and the MnPO (Sherin et al., 1996; Gong et al., 2004; Gvilia et al., 2006), which would facilitate NREMS consolidation during recovery. Another explanation is that the increased sleep drive during the recovery period overshadowed any persisting effects of E treatment that occur during the baseline period, i.e., fragmentation of NREMS.

During the second half (light phase) of recovery there were no significant differences between groups in NREMS amount, or in the number and duration of NREMS episodes. There were also no differences in NREMS in the second half of recovery in comparison with baseline values, with the exception of the Oil treated group which showed decreased NREMS episode durations during the second half of recovery. The time course of NREMS amounts, assessed in 2 h intervals, also did not differ between groups over the 24 h recovery period. These data indicate that E and E+P do not affect NREMS during the latter portion of recovery.

All groups showed REMS rebound after sleep deprivation, with no group differences. REMS episode duration tended to increase during recovery versus baseline, with this increase reaching statistical significance in the Oil treated animals. More dramatically, the number of REMS episodes approximately doubled in all treatment groups during recovery versus baseline, with E and E+P treated animals showing fewer episodes of REMS than Oil treated animals. The lower number of REMS episodes during recovery in E and E+P, compared to Oil, suggests that hormonal effects on REMS episode number persist into the recovery period, despite increased sleep drive following sleep deprivation. It should be noted that while E and E+P differed from Oil treated animals in the absolute number of REMS episodes, the percent change from baseline to recovery did not differ between groups. The similar percent changes between groups suggest a similar homeostatic response to sleep deprivation in all three groups, during the first half of the recovery phase. The increased number of REMS episodes during recovery versus baseline in the E and E+P groups implies that there is improved REMS

initiation following sleep deprivation in these groups; this may be due to improved NREMS maintenance during the recovery period.

The amount of REMS during the second half (light phase) of recovery did not differ significantly from baseline values, or between treatment groups, with the exception of the E+P treated animals, which showed elevated amounts of REMS and duration of REMS episodes, both compared to Oil treated animals and relative to the corresponding baseline period. The percent change from baseline to recovery also did not differ significantly between groups, again with the exception of the E+P group, which showed a higher percent change than the Oil treated animals. We interpret these data to suggest an increased REMS homeostatic response with E+P treatment; it should be noted that E treated animals showed a trend for increased percent change in REMS over baseline, compared to the Oil treated animals, indicating that E treatment alone may facilitate REMS recovery, but that this effect is amplified with P co-administration. The mechanisms controlling rebound increases in REMS, in response to increased homeostatic pressure, are not well understood, but are thought to be different from those regulating spontaneous REMS in the absence of sleep deprivation. The median preoptic nucleus has been proposed as a brain region involved in REMS homeostasis (Gvilia et al., 2006); this nucleus contains both ERs and PRs (Hagihara et al., 1992; Simerly et al., 1996).

The time course for REMS amounts during the dark phase did not differ between groups; however, Oil treated animals displayed a negative rebound of REMS amount during the light phase of recovery, where the amount of REMS was significantly lower than baseline values during the corresponding time period. No negative rebound in

REMS was observed during the light phase, or at any time point, for either the E or E+P treated groups. This further supports that E and E+P facilitate REMS recovery, and that these facilitatory effects of E and P are observed during the first half of recovery to some extent, but more dramatically during the second half (light phase) of recovery.

All treatment groups showed the expected increase in NREMS delta power immediately after sleep deprivation, which lasted for 4 h in all groups, although there was a trend for a lower absolute level of NREMS delta power in E and E+P treated rats compared to the Oil group during the initial 4 h. This difference in NREMS delta power between Oil versus E or E+P treated rats may be due to a mutually inhibitory interaction between NREMS intensity and REMS propensity, which was elevated in these groups (Tobler et al., 1990; Endo et al., 1997). The Oil treated group showed a trend for an earlier reduction of NREMS delta power, with mean values dropping below baseline at 6 h after sleep deprivation, i.e., midway through the first half (dark phase) of recovery. This differs from the NREMS delta power in E and E+P groups, which gradually tapered off and returned to baseline values at the start of the light phase. This implies that E and E+P may compensate for an overall reduction in absolute NREMS intensity by showing an extended elevation in NREMS delta power compared to the Oil group. When analyzed over a 12 h period, there were no increases in NREMS delta power or REMS theta power during the either first 12h (dark phase) or second 12h (light phase) of recovery.

The observed effects of E and E+P treatment on NREMS delta power during recovery are inconsistent with previously published data in women. A study of post-menopausal women reported reduced NREMS delta power after 40 h of sleep deprivation

in women on HRT consisting of E and P, compared to their untreated peers (Kalleinen et al., 2006). In contrast, while we observed an initial reduction in NREMS delta power in E and E+P groups, there was no overall reduction in NREMS delta power during recovery in comparison with Oil treated animals. The difference between these results could be due to a number of factors, including species, age (as our rats were in the peri- not post-menopausal period), choice of control group (they used a comparison group of young women) or treatment regimens (women in the study were treated with estradiol hemihydrate and norethisterone acetate taken orally). E+P treated animals did show decreased delta power and increased gamma power during wake episodes compared to Oil treated animals. It is interesting to note that the increased gamma power during wake with E+P treatment, but not E alone, occurred both during baseline and recovery and that this effect was only observed in the dark (active) phase. The temporal gating of these EEG effects suggests involvement of circadian mechanisms.

A previous study in our laboratory by Deurveilher et al. (2009, 2011) also examined the effects of ovarian hormones on 18h of recovery following 6 h of sleep deprivation, in ovariectomized young female rats (Table 8). They found that all groups showed NREMS and REMS rebound during the dark phase (first 12h) of recovery, and that the absolute amounts of NREMS and REMS during this period were similar for all treatment groups. The number and duration of NREMS episodes was increased above baseline in the LE, HE and LEP groups. However, the percent change in REMS amount from baseline to recovery was enhanced in all hormonally treated rats (LE, LEP and HE) compared with Oil treated rats. The number of REMS episodes, in all groups, and the mean duration of REMS episodes, in the HE and LEP groups, also showed a rebound

increase; however, the absolute number of REMS episodes during recovery was lower in both HE and LEP groups in comparison with Oil treatment. While the absolute number of REMS was lower in HE and LEP groups, the percent change from baseline was higher, although not significantly so, in the HE and LEP groups compared to the Oil group. The percent change in NREMS and REMS episode durations was significantly greater in the HE and LEP groups compared to the Oil group.

Deurveilher et al. (2009, 2011) also observed an increase in NREMS delta power during the first 4 h of recovery and that E and P replacement reduced both the initial level and duration of increase of NREMS delta power, compared to Oil treated animals. Furthermore, animals treated with E alone, at both high (HE) and low doses (LE), showed a negative rebound in NREMS delta power late in recovery (generally at the start of the light phase); this effect was abolished with concomitant treatment with P.

The general trends were similar between the present study and those reported by Deurveilher et al. (2009, 2011) using middle-aged and young female rats, respectively, including rebound increases in NREMS and REMS amounts during recovery for all groups, increased number of NREM and REMS episodes during recovery for all groups, and the elevation of NREMS delta power in the first 4h of recovery for all groups. In particular, both studies found increased REMS rebound in response to E treatment. A summary of the comparisons between middle-aged and young animals can be seen in Table 8.

There are a number of possible explanations for the effects of E treatment. For example, the promotion of REMS rebound could be due to direct effects of E on brain nuclei involved in REMS homeostasis, such as the MnPO (Gvilia et al., 2006). Both

studies showed an increased proportion of REMS in the light phase at baseline. Thus, E treated animals could have increased REMS drive during recovery as a larger proportion of daily REMS would have been lost during sleep deprivation, which occurred during the light phase. It is also conceivable that the inhibitory effect of E on REMS initiation that is normally at work during baseline recording was overpowered by the increased homeostatic pressure the animals experienced during the recovery period. Which explanation, or combination thereof, is actually responsible is unclear; however, further investigations are warranted.

There were several interesting differences in the effects of hormonal treatment between the middle-aged animals used in the present study and the young animals used by Deurveilher et al (2009, 2011). First, the effects we observed with E treatment continued to be most similar to the HE treatment group, not the LE group, during the recovery phase; this was also the case during the baseline recordings. This indicates that there may be an increase in potency of E treatment in middle-aged versus young animals. Second, while we observed no differences between groups in the duration of NREMS episodes during recovery, with all groups showing increased NREMS episode durations, Deurveilher et al. (2009, 2011) found that only hormonally treated rats (LE, HE, LEP groups), but not the Oil group, showed this increase in NREMS episode duration during recovery. A summary of the comparisons between middle-aged and young animals can be seen in Table 8. The origin of this difference between the Oil treated rats in these studies is not known, although it could be due to a number of factors which differed between these studies, including animal age.

There were also differences in NREMS delta power during recovery in the middle-aged animals in our study in comparison with young animals reported by Deurveilher et al. (2009, 2011). Both studies found that hormonally treated rats (the E, E+P, LE, HE, LEP groups) showed a trend for decreased absolute levels of NREMS delta power at the start of recovery. However, we found that the duration of NREMS delta power elevation was extended in E and E+P treated animals, whereas they found that treatment with ovarian hormone (LE, HE, LEP groups) curtailed the duration of the NREMS delta power rebound. They also found that E treated animals, both the LE and HE groups, had a negative rebound in NREMS delta power early in the latter portion of recovery (light phase); we did not observe any such effect despite recording for an additional 6 h. It is interesting to note that we found that E and E+P treated middle-aged rats compensate for decreased absolute intensity of NREMS, by sustaining the elevation in NREMS sleep delta power longer than Oil treated rats; in contrast, young animals receiving ovarian hormones showed impairments in both the absolute level and the duration of rebound intensity increases. The mechanisms controlling NREMS homeostasis have not been fully elucidated; however, the basal forebrain has been shown to play a role in sleep recovery following sleep loss and the thalamocortical system has been shown to be involved in the generation of slow waves (Hill & Tononi, 2005; Kalinchuck et al., 2008; Kaur et al., 2009); it is possible that E and P could directly influence homeostatic regulation and slow wave generation in these regions, as they contain ERs and PRs (Kato et al., 1994; Shughrue et al., 1997; Saper et al., 2010).

It is unlikely that a reduction in sleep pressure was responsible for the decreased initial sleep intensity observed in both age groups in the E and P treated animals, because

the number of interventions required to keep animals awake during sleep deprivation did not differ between treatment groups in both studies. However, it is possible that differences in sleep drive underpin the differences between the two age groups; the middle-aged animals with Oil and E treatment tended to require more frequent interventions in the 6th hour of sleep deprivation than the young counterparts (Deurveilher et al., 2009, 2011). The observed differences in NREMS delta power between age groups could also be due to changes in ER or PR expression patterns with aging, differences in receptor subtype expression, or due to unknown differences; however, it is clear that there are differences, as well as similarities, between sleep/wake patterns in response to hormonal treatment in young versus middle aged animals, both at baseline and after sleep deprivation.

2.4.3 Methodological Issues

There are a number of issues that must be addressed when considering the implications of the present results. First, we used ovariectomized female rats with subcutaneous steroid implants, as we wanted a stable background (2.5 days) against which to compare hormonal effects. This cannot be accomplished in intact rats due to the rapid changes in hormone levels associated with the short duration of estrous cycle stages. However, as ovarian hormone production is regulated by a negative feedback loop along the hypothalamus-pituitary-ovarian axis, the removal of estrogen should result in elevated plasma levels of gonadotropin in the Oil group (Crowley et al., 1978; Shupnik et al., 1988). The effects of gonadotropin on sleep/wake regulation are not currently known; however, previous studies have shown that ovariectomized rats show higher

amounts of REMS than intact rats at any stage of the estrous cycle (Fang & Fishbein, 1996; Li & Santinoff, 1996). Thus, it is possible that the observed differences between hormonally treated rats and our control group may be due to E's effect on gonadotropin levels, rather than a direct effect of E and P on sleep/wake regulation.

Secondly, there is some debate as to what is the appropriate age in rats to model menopause. Menopause in humans is accompanied by reduced circulating E, loss of menses and reproductive ability; this is due to the loss of ovarian follicles (Gosden & Faddy, 1994; Burger, 1996). The peri-menopausal period is characterized by irregular hormonal cycles, and women are considered to be post-menopausal when they are reproductively acyclic. These changes with reproductive aging appear to be unique to humans and some higher non-human primates. Rodents experience reductions in fertility and cessations of reproductive cycles; however, this is not accompanied by changes in the ovaries or reductions in follicular stores, nor is it accompanied by declining E levels (Chakraborty & Gore, 2004). Rats tend to show irregular, and often prolonged, estrous cycles between 9-12 months of age; this is similar to the peri-menopausal period in humans. They usually transition to acyclicity at around 12 months and enter a phase called persistent estrus, which is characterized by chronically high (proestrus) levels of E. This phase is followed by persistent diestrus, which is characterized by unremitting, low levels of E (Lu et al., 1979; LeFevre & McClintock, 1988). These inter-species differences in reproductive aging complicate choosing the appropriate age for modeling the human menopause in rodents, and the differences in circulating E levels makes OVX a necessary protocol component.

We chose to study the effects of ovarian hormones in middle-aged, "peri-menopausal" rats; however, it could be debated that rats that have ceased to show estrous cycle-related hormonal fluctuations (i.e. post-menopausal) would provide a better model system. Were we to use post-menopausal rats we could study the effects of ovarian hormone replacement in OVX rats; this could be argued to better represent naturally menopausal women who often either begin or continue HRT into the post-menopausal period. However, we chose to use peri-menopausal rats for two reasons; first, current clinical treatment recommendations are to begin treatment as soon as possible, preferably in the peri-menopausal period, and human studies have shown that delays in initiation of HRT increase the risk of adverse events (NAMS, 2010). Second, using post-menopausal rats would induce greater age variability, as not all animals would cease to cycle at the same age, thus introducing a new confounding factor in data interpretation. We felt these reason justified our choice of age group, although it would be interesting to see if there were differences in sleep/wake patterns in post- versus peri-menopausal animals.

2.4.4 Conclusions

We show for the first time that E replacement affects recovery following sleep loss in ovariectomized middle-aged female rats, by extending NREMS delta power elevation and promoting REMS recovery, and that co-administration of P further enhances REMS recovery. We also showed that E and P influence spontaneous sleep patterns in middle-aged rats by increasing NREMS fragmentation and reducing REMS initiation. It is also evident from these results, that the effects of ovarian hormones during baseline sleep do not mirror the effects observed during recovery from sleep

deprivation. These results suggest that E and P may have both combined and independent effects on sleep/wake regulation and support the conclusion that HRT may have direct effects on sleep patterns in menopausal women.

CHAPTER 3 - DISCUSSION

3.1 CLINICAL IMPLICATIONS

The effects of E and P on NREMS maintenance during spontaneous sleep are biologically relevant, as deficits in NREMS maintenance could affect how well rested individuals feel on a daily basis. Impaired REMS initiation could also affect an individual's quality of life, as both REMS and NREMS have been proposed to contribute to memory consolidation (Diekelmann & Born, 2010). The facilitation of REMS recovery following sleep deprivation may also be relevant to general populations, as sleep loss is extremely common among people in modern societies (Broman et al., 1996) and the ability to recover following sleep loss is important for both mental and physical well being.

The reduction in spontaneous sleep quality that we observed in our hormone treated rats may help explain the conflicting results in human studies regarding the effect of HRT on sleep (Polo-Kantola, 2011). If the impairments we observed in rats also occur in human subjects, there would be two opposing effects of HRT on sleep. On one hand, in symptomatic women, HRT would improve sleep quality indirectly by reducing the number and intensity of hot-flashes, which themselves would contribute to sleep fragmentation and a reduction in an individual's overall sense of sleep quality. On the other hand, the direct hormonal effects on sleep appear to increase NREMS fragmentation, thus reducing overall sleep quality. Therefore, there could be an overall net effect of no improvement in sleep quality if hormone induced sleep disruption equals the reduction in hot-flash induced sleep disruption. However, in women with more

severe climacteric symptoms, there may be an overall improvement in sleep quality, and women with mild climacteric symptoms would experience reduced sleep quality.

Our results may also help explain the differences between subjective and objective reports of the effects of HRT on sleep in human studies (Polo-Kantola, 2011). The observed fragmentation of NREMS occurred primarily during the dark (active) phase in our middle-aged rats, which could be due to increased arousal in these rats during their active phase. This could also impact subjective, but not objective, measures of sleep quality, since there would be a decreased feeling of daytime sleepiness, which is an important component of subjectively determining sleep quality. If this is case, HRT would be beneficial in improving the quality of life in peri and post-menopausal women.

Extrapolating from rodent studies to human populations should be done with care. Many factors can contribute to the differences observed between treatment groups, as we have previously discussed. There are also many inter-species differences in sleep regulation, the most readily apparent difference being that rodents are primarily nocturnal and show a polyphasic sleep pattern, as opposed to the a single consolidated sleep period in the dark phase, which most humans experience. A better understanding of the mechanisms involved in sleep/wake regulation, as well as the interspecies differences, is necessary before we can apply these results to help interpret the effect of HRT on sleep in menopausal women.

3.2 FUTURE STUDIES

3.2.1 Comparison of effects of ovarian hormones in peri- and post-menopausal rats

Many women continue HRT after they enter the post-menopausal period. Sleep quality and quantity impact many aspects of quality of life, including mood, memory, and attention (Polo-Kantola, 2011). Decreased sleep quality and quantity has also been linked to increased risk for various health problems (Knutson & Van Cauter, 2008). While the results of our study improve our understanding of the effects of ovarian hormone on the peri-menopausal period, we do not know whether our findings can be applied to the post-menopausal period. A study which directly compared the effects of E and E+P in both peri- (10-11 mos) and post-menopausal (13-14 mos) rats, both during spontaneous sleep and during recovery from sleep deprivation could help elucidate the interactions between aging and female sex hormone on sleep.

3.2.2 The effects of ovarian hormones in middle-aged rats using a chronic sleep restriction paradigm

Chronic sleep restriction is incredibly common in industrialized countries, due to increasing demand to work extended hours and at non-traditional times (Broman et al., 1996); however, the frequency of acute total sleep loss is much less common. Whether the homeostatic control of recovery sleep and effects of acute sleep loss are the same as the homeostatic control of recovery sleep and effects of chronic restriction is not clear. It is likely that the effects of ovarian hormones may differ in the context of chronic sleep

restriction versus acute total sleep deprivation. Thus, I would propose to study the effects of ovarian hormones in ovariectomized middle-aged female rats during chronic sleep restriction using the "3/1" chronic restriction protocol developed in our laboratory. This protocol induces 3h of wake, by using slowly rotating wheels, followed by a 1h sleep opportunity window, where the wheel remains motionless. This protocol continues over a 4 day period followed by 2 days of recovery. This study could help elucidate the role of ovarian hormones and age in sleep/wake regulation in a sleep loss model that is more relevant to people.

3.2.3 The effects of ovarian hormones in middle-aged rats using a sleep fragmentation paradigm

Studies of the effects of HRT on sleep are complicated in human studies by the presence of hot-flashes, which can directly impair sleep quality. While HRT can ameliorate climacteric symptoms, they are often not completely abolished (Polo-Kantola, 2011). A rodent model could help study the interaction of hot-flash induced sleep-fragmentation and ovarian hormones on sleep quality, and whether the homeostatic response to sleep fragmentation differs from spontaneous sleep patterns or recovery sleep following sleep deprivation. An activity wheel can be used to produce 30 short awakenings per hour, over a 4 day period; this model has been used in previous experiments examining the effects of sleep apnea (McCoy et al., 2007). The use of activity wheels, or another method, to induce sleep fragmentation is necessary as rats do not naturally experience hot-flashes or the sleep disruptions associated with them (Pawlyk et al., 2008a, 2008b).

CHAPTER 4 - CONCLUSION

Both animal and human studies indicate a role for ovarian hormone in sleep/wake regulation, but what part they play remains unclear. The use of ovarian hormones in HRT increases our need to understand the effects of these hormones in older populations. Improving our understanding of sleep/wake regulation and the effects of ovarian hormones in the peri-menopausal period, can help to clarify the effects of E and P in menopausal women and could, potentially, help us improve quality of life both during and after the menopausal transition.

Table 1. Wake-promoting nuclei and the density of estrogen and progesterone receptors in these regions of the rat brain.

Brain Area	NT	Receptor Density			Projection Targets	Actions at Target
		ERα	ERβ	PR		
Wake Promoting						
LDT	ACh	-	+	++	Thalamus VLPO, MnPO	Excitatory Inhibitory
PPT	ACh	-	+	+	Thalamus VLPO, MnPO	Excitatory Inhibitory
LC	NE	+	+	++++	BF, Hypothal, Cerebral cortex VLPO, MnPO, SLD, PC, LDT, PPT	Excitatory Inhibitory
PB	Glu	+	+	++	BF, Hypothal, Cerebral cortex VLPO, MnPO	Excitatory Inhibitory
PC	Glu	+	+	-	BF, Hypothal, Cerebral cortex VLPO, MnPO	Excitatory Inhibitory
DR	5-HT	-	+	+++	BF, Hypothal, Cerebral cortex VLPO, MnPO, SLD, PC, LDT, PPT	Excitatory Inhibitory
vPAG	DA	++	+	+++	BF, Hypothal, Cerebral cortex VLPO, MnPO	Excitatory Inhibitory
TMN	HA	-	++	+	BF, Hypothal, Cerebral cortex VLPO, MnPO	Excitatory Inhibitory
LH	Orexin	+	+	++++	BF, Cerebral cortex, Brainstem	Excitatory
Projection Targets						
Thal	Glu	+	+	+/-		
BF	GABA ACh	+	++	++/-		

Abreviations: NT: neurotransmitter; ER-α: estrogen receptor α; ER-β: estrogen receptor β; PR: progesterone receptor; ACh: acetylcholine; DA: dopamine; Glu: glutamate; HA: histamine; NE: norepinephrine; 5-HT: serotonin; BF: basal forebrain; DR: Dorsal raphe nucleus; Hypothal: hypothalamus; LC: Locus coeruleus; LDT: laterodorsal tegmental nucleus; LH: Lateral hypothalamus; LPT: lateral pontine tegmentum; MnPO: median preoptic area; MR: midbrain raphe nucleus; PB: Parabrachial nucleus; PC: Precoeruleus nucleus; PPT: Pedulopontine tegmental nucleus; SLD: sublaterodorsal region; Thal: Thalamus; TMN: Tubermammilary nucleus; VLPO: ventrolateral preoptic area; vPAG: Ventral periaqueductal gray

Adapted from Saper et al., 2010; Shughrue et al., 1997; Kato et al., 1994.

Table 2. Sleep-promoting nuclei and the density of estrogen and progesterone receptors in these regions of the rat brain.

Brain Area	NT	Receptor Density			Projection Targets	Actions at Target
		ERα	ERβ	PR		
NREM Promoting						
VLPOc	GABA	+	+	+	TMN, LH, MR, LC, BF	Inhibitory
MnPO	GABA	++++	++++	+	LH, LC, DR, vlPAG, SLD	Inhibitory
REM Promoting						
SLD	GABA Glu	-	+	-	vlPAG, LPT PB, PC	Inhibitory Excitatory
PC	Glu	+	+	-		
LDT	ACh	-	+	++	SLD, PC vlPAG, LPT	Excitatory Inhibitory
PPT	ACh	-	+	-	SLD, PC vlPAG, LPT	Excitatory Inhibitory
VLPOe	GABA	+	+	+	vlPAG, LPT, DR, LC, LDT	Inhibitory
REM Inhibiting						
DR	5-HT	-	+	+++	LDT, PPT, SLD, PC vlPAG, LPT	Inhibitory Excitatory
LC	NE	+	+	++++	LDT, PPT, SLD, PC vlPAG, LPT	Inhibitory Excitatory
LH	Orexin	+	+	+	vlPAG, LPT	Inhibitory
Circadian Regulation						
SCN	GABA	+++	+++			

Abreviations: NT: neurotransmitter; ER-α: estrogen receptor α; ER-β: estrogen receptor β; PR: progesterone receptor; ACh: acetylcholine; DA: dopamine; Glu: glutamate; NE: norepinephrine; 5-HT: serotonin; BF: basal forebrain; DR: Dorsal raphe nucleus; LC: Locus coeruleus; LDT: laterodorsal tegmental nucleus; LH: Lateral hypothalamus; LPT: lateral pontine tegmentum; MnPO: median preoptic area; MR: midbrain raphe nucleus; PB: Parabrachial nucleu; PC: Precoeruleus nucleus; PPT: Pedulopontine tegmental nucleus; SCN: suprachiasmatic nucleus; SLD: sublaterodorsal region; TMN: Tubermammilary nucleus; vlPAG: ventero lateral periaqueductal gray; VLPOc: ventrolateral preoptic area (cluster); VLPOe: ventrolateral preoptic area (extended); vPAG: Ventral periaqueductal gray

Adapted from Saper et al., 2010; Shughrue et al., 1997; Kato et al., 1994.

Table 3. Summary of the findings of recent studies on the effects of hormonal replacement in ovariectomized young adult female rats on baseline sleep and recovery sleep following 6 h of total sleep deprivation (Deurveilher et al., 2009; Deurveilher et al., 2011).

Sleep parameters	Treatment groups
	Young
Baseline (24 h)	
NREMS amount (min)	HE, LEP < Oil, LP
REMS amount (min)	LE, HE, LP < Oil
Baseline (12h light)	
REMS amount (min)	LE, HE, LP < LEP
Baseline (12h dark)	
REMS amount (min)	HE, LP, LEP < Oil
NREMS episodes duration (s)	HE, LP, LEP < Oil
REMS episodes number	HE, LEP < Oil and LEP < LP
Recovery (12 h dark)	
NREMS amount (% baseline dark)	-
NREMS episode number	-
NREMS episode duration (% baseline dark)	HE, LEP > Oil
REMS amount (% baseline dark)	HE, LEP > Oil
REMS episode number	HE, LP, LEP > Oil
REMS episode duration (% baseline dark)	HE, LEP > Oil
NREM EEG delta activity in first 2 h	HE, LP, LEP < Oil

Rats were ovariectomized and implanted subcutaneously with Silastic tubing containing oil vehicle, 17β-estradiol (E), progesterone (P), or both E and P. Animals were treated with either high (H) or low (L) doses of the hormones.

After 2 weeks, sleep/wake states were recorded during a 24-h baseline period, during 6 h of total sleep deprivation induced by gentle handling in the light phase, and during an 18-h recovery period.

↑ increase; ↓ decrease; - no effect.

Adapted from Deurveilher et al., 2011.

Table 4. Hormone amounts per capsule and resulting serum levels in young and middle-aged female rats.

Treatment Groups	Hormone amounts per capsule	Serum hormone levels	Corresponding day of the estrous cycle	References
Young				
OVX + Oil	-	E: < 5 pg/ml P: < 2 ng/ml	n/a	Dubal & Wise, 2001; Deurveilher et al., 2008; 2009
OVX + E	E: 10.5 µg	E: 10-15 pg/ml	Diestrus	Dubal & Wise, 2001; Deurveilher et al., 2008; 2009
OVX + E+P	E: 10.5 µg P: 40 mg	E: 10-15 pg/ml P: 3-5 ng/ml	Diestrus	Dubal & Wise, 2001; Deurveilher et al., 2008; 2009
Middle-Aged				
OVX + Oil	-	E: < 5 pg/ml P: < 2 ng/ml	n/a	Dubal & Wise, 2001
OVX + E	E: 14 µg	E: 10-15 pg/ml	Diestrus	Dubal & Wise, 2001
OVX + E+P	E: 14 µg P: 50 mg	E: 10-15 pg/ml P: 3-5 ng/ml	Diestrus	Dubal & Wise, 2001; Mannino et al., 2005

Steroid capsules were made of Silastic tubing (outer diameter = 3.2 mm; inner diameter = 1.6 mm) with lengths of 35 mm for E and 40 mm for E+P in young rats, and 45 mm for E and 50 mm for E+P in middle-aged rats. The different lengths (i.e., different E/P amounts) account for differences in the body weight between the young and middle-aged groups (Dubal & Wise, 2001 [55]). E was dissolved in sesame oil; P was used in crystalline form. The amounts of E and P in the current study are similar to those previously used in the Low E, Low P and Low E+P groups (Deurveilher et al., 2009 [48]; Table 1) to produce physiological levels of the hormones as observed during natural estrous cycles.

Abbreviations: E, estradiol; OVX, ovariectomized; P, progesterone.

Adapted from Semba K, Rusak B, Deurveilher S (2009) CIHR grant application.

Table 5. Plasma Estradiol and Progesterone Levels, Uterus Weights, and Body Weights.

Variables	Treatment groups		
	Oil (5)	**E (5)**	**E+P (5)**
Plasma estradiol level (pg/mL)	< 5	14.7	14.6
Plasma progesterone level (ng/mL)	1.1 ± 0.22	2.2 ± 0.9	8.4 ± 1.7*
Uterus weight (mg)	199.0 ± 11.7	687.3 ± 59.3*	540.0 ± 52.2*
Uterus weight/body weight ratio (x 10^3)	0.55 ± 0.03	1.92 ± 0.15*	1.66 ± 0.14*
Body weight			
At surgery (g)	418.8 ± 26.7	408.4 ± 18.0	373.6 ± 24.2
At sacrifice (g)	366.0 ± 17.9	357.3 ± 11.5	325.8 ± 16.9
Change in body weight (g)	-52.4 ± 10.6	-51.1 ± 9.6	-47.8 ± 14.7

All values are expressed as mean ± SEM, except for plasma estradiol levels for which the median is given, as the data contained values below the detection limit of the assay (5 pg/mL). The numbers of animals are indicated in brackets. * Different from Oil treatment group; all $P < 0.05$. Fisher LSD post hoc tests were used for all variables except for plasma estradiol levels, which were analyzed using nonparametric Dunn post-hoc tests.

Table 6. Mean duration (sec) of Episodes of Wake, NREM and REM Sleep during Baseline Sleep and Recovery Sleep after Sleep Deprivation.

Stage, Group, Condition.	24 h Total	12 h Light	12 h Dark
Wake			
Oil			
Baseline	112 ± 8	47 ± 3	249 ± 15
Recovery	90 ± 8#	47 ± 5	147 ± 8#
E			
Baseline	132 ± 22	60 ± 13	295 ± 69
Recovery	110 ± 9	47 ± 4	197 ± 22
E+P			
Baseline	117 ± 13	47 ± 2	311 ± 68
Recovery	100 ± 5	46 ± 1	175 ± 33#
NREMS			
Oil			
Baseline	90 ± 6	95 ± 7	79 ± 4
Recovery	86 ± 7	79 ± 8#	105 ± 10#
E			
Baseline	76 ± 9	86 ± 9	52 ± 6*
Recovery	81 ± 11	83 ± 10	82 ± 14 #
E+P			
Baseline	73 ± 7	79 ± 7	58 ± 4*
Recovery	82 ± 5	83 ± 8	83 ± 6 #
REMS			
Oil			
Baseline	57 ± 9	59 ± 10	55 ± 5
Recovery	59 ± 10	51 ± 9	67 ± 10#
E			
Baseline	66 ± 13	63 ± 12	79 ± 19
Recovery	74 ± 12	62 ± 11	102 ± 16
E+P			
Baseline	64 ± 5	63 ± 5	65 ± 8
Recovery	78 ± 7	72 ± 6 #	83 ± 12

All values are expressed as mean ± SEM. * Different from Oil Treated group. #Different from corresponding baseline. All $P < 0.05$; Fisher LSD post hoc comparisons were performed to analyze effects between treatment groups and paired t-tests for effects within treatment groups. Recovery started at the beginning of the dark phase.

Table 7. Mean Number of Episodes of Wake, NREM and REM Sleep during Baseline Sleep and Recovery Sleep after Sleep Deprivation.

Stage, Group, Condition.	24 h Total	12 h Light	12 h Dark
Wake			
Oil			
Baseline	392 ± 27	267 ± 22	125 ± 5
Recovery	410 ± 40	314 ± 40	149 ± 8 #
E			
Baseline	413 ± 51	274 ± 29	140 ± 23
Recovery	392 ± 32	295 ± 32	151 ± 19
E+P			
Baseline	444 ± 53	315 ± 30	129 ± 23
Recovery	411 ± 34	298 ± 25	163 ± 21 #
NREMS			
Oil			
Baseline	419 ± 18	289 ± 16	130 ± 4
Recovery	508 ± 26 #	341 ± 23	167 ± 17 #
E			
Baseline	423 ± 46	287 ± 23	136 ± 23
Recovery	469 ± 30	320 ± 29	149 ± 19
E+P			
Baseline	448 ± 53	319 ± 30	128 ± 24
Recovery	469 ± 40	304 ± 23	165 ± 23 #
REMS			
Oil			
Baseline	125 ± 33	86 ± 30	38 ± 4
Recovery	157 ± 43	80 ± 31	77 ± 13 #
E			
Baseline	87 ± 19	70 ± 16	17 ± 5*
Recovery	122 ± 39	86 ± 33	36 ± 8* #
E+P			
Baseline	90 ± 7	66 ± 8	24 ± 6*
Recovery	114 ± 10#	68 ± 10	47 ± 4* #

All values are expressed as mean ± SEM. * Different from Oil Treated group. #Different from corresponding baseline. All $P < 0.05$; Fisher LSD post hoc comparisons were performed to analyze effects between treatment groups and paired t-tests for effects within treatment groups. Recovery started at the beginning of the dark phase.

Table 8. Comparison of the findings of the current study with recent studies on the effects of hormonal replacement in ovariectomized young female rats (Deurveilher et al., 2009; Deurveilher et al., 2011) **on baseline sleep and recovery sleep following 6 h of total sleep deprivation.**

Sleep parameters	**Treatment groups**	
	Young	**Middle-Age**
Baseline (24 h)		
NREMS amount (min)	HE, LEP < Oil, LP	E, E+P < Oil
NREMS amount L/D ratio	*Oil < LE, HE < LEP*	*Oil < E, E+P*
REMS amount (min)	LE, HE, LP < Oil	----------
REMS amount L/D ratio	Oil, LE < HE, LEP	*Oil < E, E+P*
Baseline (12h light)		
REMS amount (min)	LE, HE, LP < LEP	----------
Baseline (12h dark)		
NREMS amount (min)	*LEP, HE < LE, Oil*	E, E+P < Oil
NREMS episode duration (s)	HE, LEP < Oil < LP	E, E+P < Oil
REMS amount (min)	HE, LP, LEP < Oil	----------
REMS episode duration (s)	*HE, LEP < LE, Oil*	Oil < E, E+P
REMS episode number	HE, LP, LEP < Oil	*E < E+P < Oil*
Recovery (12 h dark)		
NREMS amount (min)	*LEP, HE, LE < Oil*	E < *E+P* < Oil
NREMS amount (% baseline dark)	Not reported	----------
NREMS episode number	*LEP < HE, LE, Oil*	----------
NREMS episode duration (s)	----------	*E, E+P < Oil*
NREMS episode duration (% baseline dark)	Oil < HE, LEP	----------
REMS amount (min)	*LEP, HE, LE < Oil*	*E < E+P, Oil*
REMS amount (% baseline dark)	Not reported	*Oil, E+P < E*
REMS episode number	Oil < HE, LP, LEP	E, E+P < Oil
REMS episode duration (s)	*Oil < HE, LEP*	*Oil, E+P < E*
REMS episode duration (% baseline dark)	Oil < HE, LEP	----------
NREM EEG delta activity in first 2 h	HE, LP, LEP < Oil	E, E+P < Oil

Rats were ovariectomized and implanted subcutaneously with Silastic tubing containing oil vehicle, 17β-estradiol (E), progesterone (P), or both E and P. Animals were treated with either high (H) or low (L) doses of the hormones.

↑ increase; ↓ decrease; - no effect.; non-significant trends are italicized.

This table has been adapted from Deurveilher et al.(2011).

Table 9. Comparison of spontaneous sleep patterns in middle-aged (current study) and young (Deurveilher et al., 2009; Deurveilher et al., 2011) **ovariectomized oil treated female rats.**

Sleep parameters	Light Phase	Dark Phase
NREMS		
Amount (min)	Y < M	Y > M
Number of episodes	Y < M	Y = M
Duration of episodes (s)	Y = M	Y > M
REMS		
Amount (min)	Y > M	Y > M
Number of episodes	Y < M	Y = M
Duration of episodes (s)	Y >> M	Y > M

Rats were ovariectomized and implanted subcutaneously with Silastic tubing containing oil vehicle.

Comparisons were made between young (Y) and middle-aged (M) female rats.

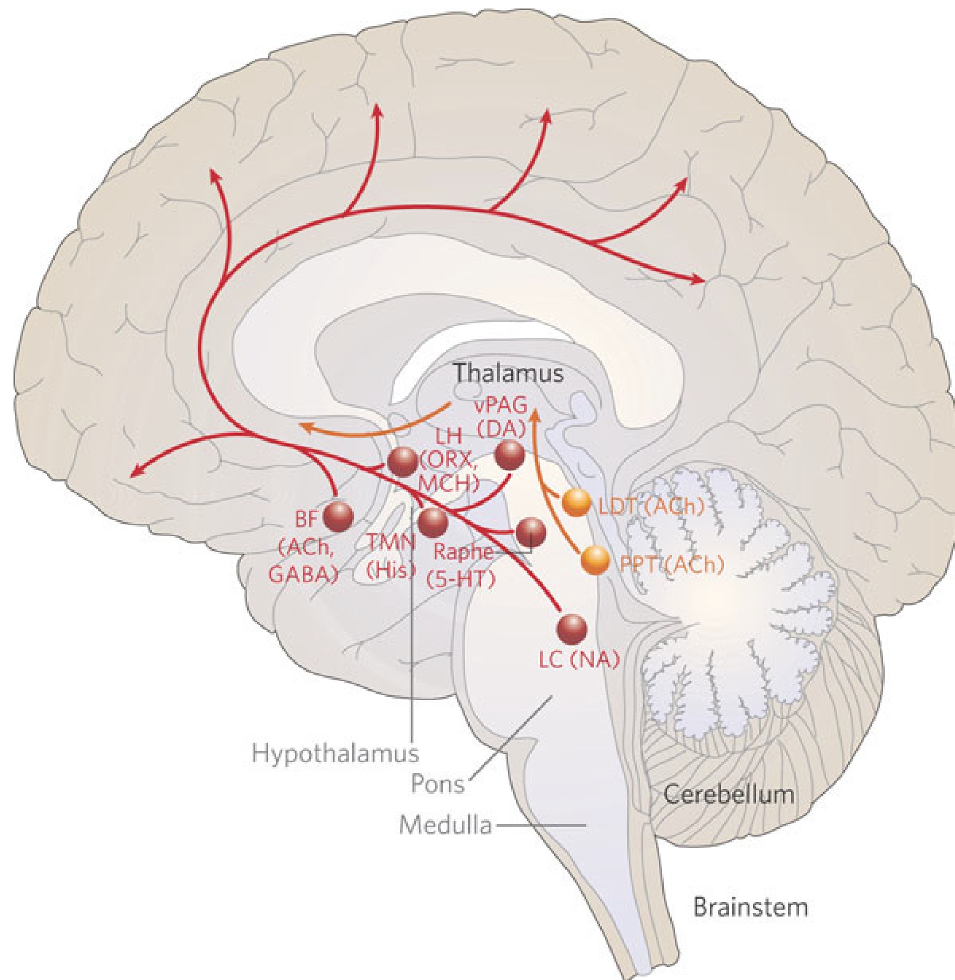


Figure 1. A schematic drawing showing key components of the ascending arousal system. A major input to the relay and reticular nuclei of the thalamus (yellow pathway) originates from cholinergic (ACh) cell groups in the upper pons, the pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT). These inputs facilitate thalamocortical transmission. A second pathway (red) activates the cerebral cortex to facilitate the processing of inputs from the thalamus. This arises from neurons in the monoaminergic cell groups, including the tuberomammillary nucleus (TMN) containing histamine (His), the A10 cell group containing dopamine (DA), the dorsal and median raphe nuclei containing serotonin (5-HT), and the locus coeruleus (LC) containing noradrenaline (NA). This pathway also receives contributions from peptidergic neurons in the lateral hypothalamus (LH) containing orexin (ORX) or melanin-concentrating hormone (MCH), and from basal forebrain (BF) neurons that contain γ-aminobutyric acid (GABA), ACh, or Glu.

Figure used with permission, from Saper et al., 2005

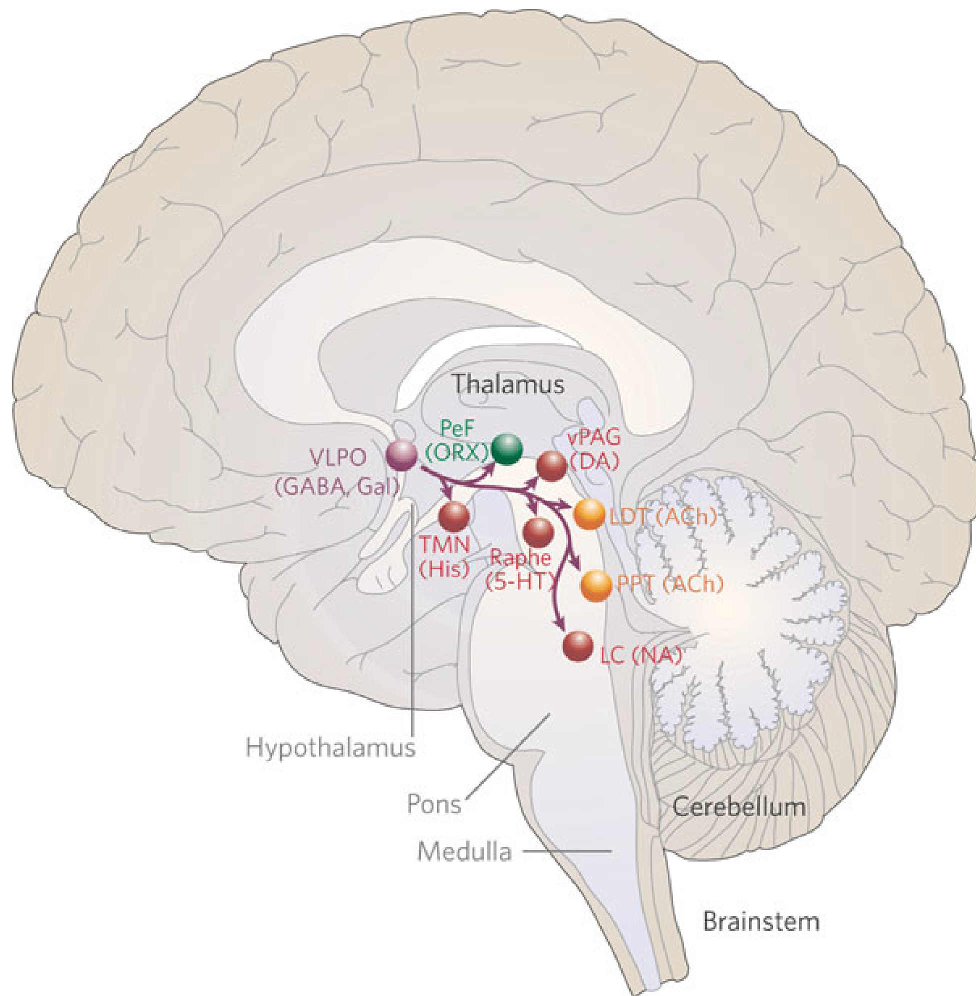


Figure 2. A schematic drawing to show the key projections of the ventrolateral preoptic nucleus (VLPO) to the main components of the ascending arousal system. Target regions include the monoaminergic cell groups (red) such as the tuberomammillary nucleus (TMN), the A10 cell group, the raphe cell groups and the locus coeruleus (LC). The VLPO also innervates neurons in the lateral hypothalamus, including the perifornical (PeF) orexin (green, ORX) neurons, and the cholinergic (ACh) cell groups (yellow), including the pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT). 5-HT, serotonin; GABA, γ-aminobutyric acid; gal, galanin; NA, noradrenaline; His, histamine.

Figure used with permission, from Saper et al., 2005

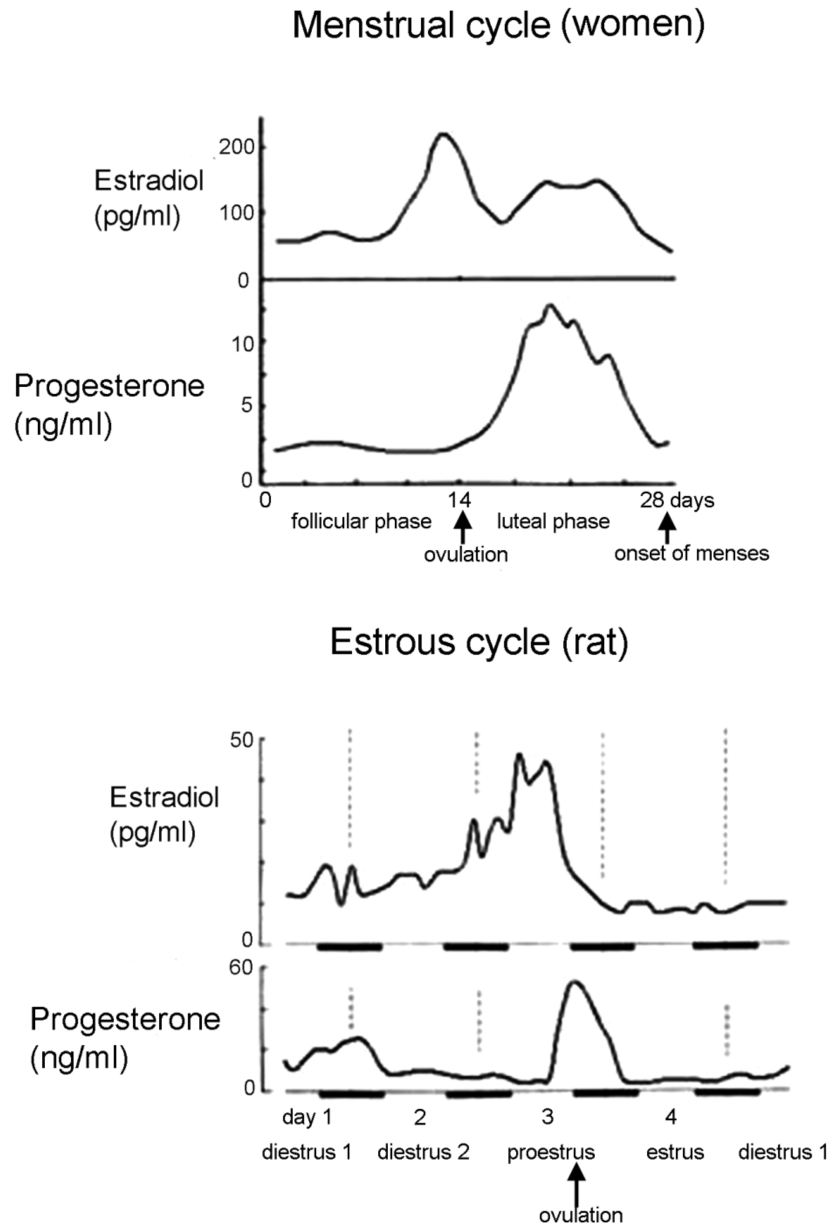


Figure 3. Changes in the plasma levels of female sex steroids during the menstrual and estrous cycles.

Adapted from Scharfman and Maclusky, 2006.

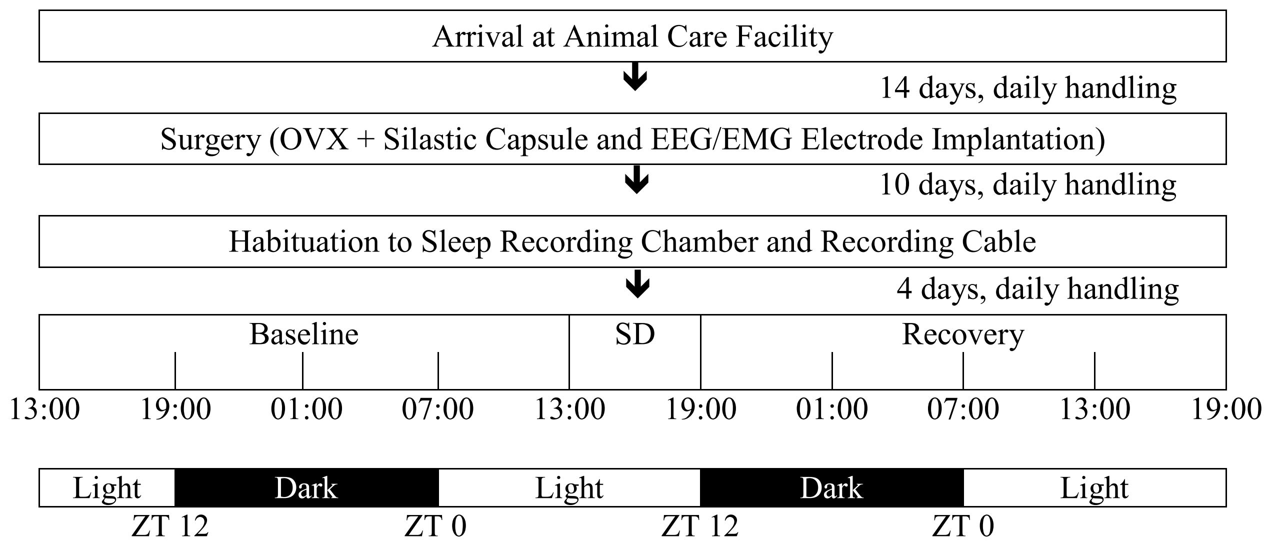


Figure 4. Experimental Procedures. SD – sleep deprivation was accomplished through gentle handling. EEG - electroencephalogram; EMG - electromyogram; OVX - ovariectomy; ZT - Zeitgeber time.

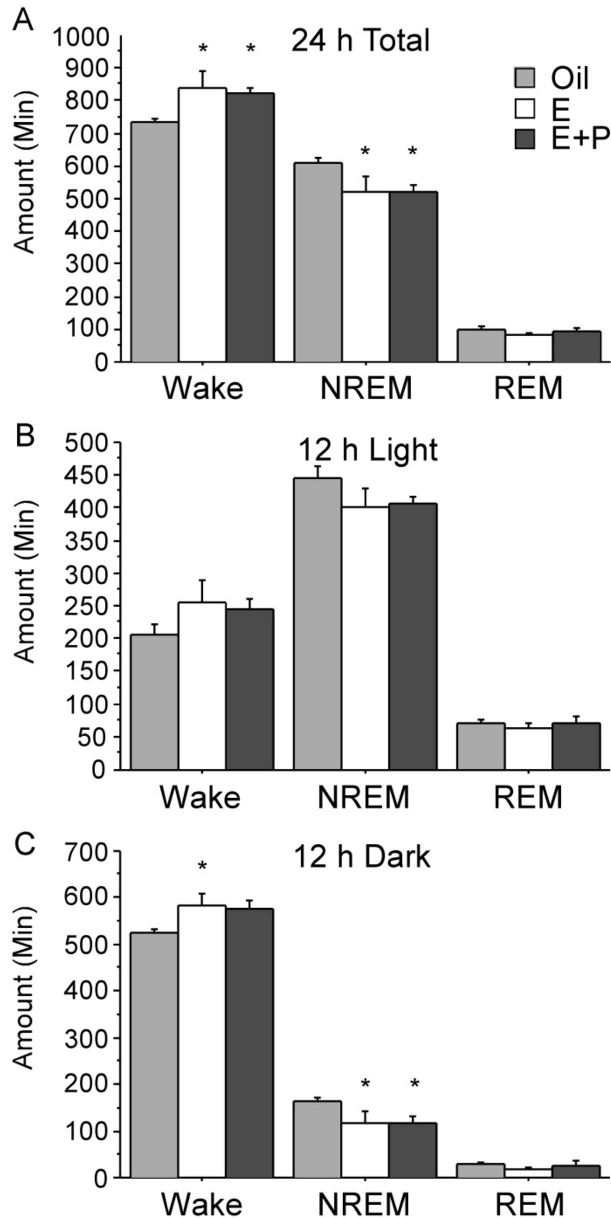


Figure 5. Amount of Wake, NREMS, REMS during baseline recording for the full 24 h period (A), 12 h light phase (B) and the 12 h dark phase (C) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). Animals treated with E, either alone or in combination with P, displayed increased wake, with a complementary decrease in NREMS, both over the 24 h period (A) and during the dark phase (C). The E treated animals also showed elevated amounts of wake during the dark phase (C). Similar trends observed in the light phase were not significant. * Different from Oil group; all $P < 0.05$ (Fisher LSD post hoc comparisons).

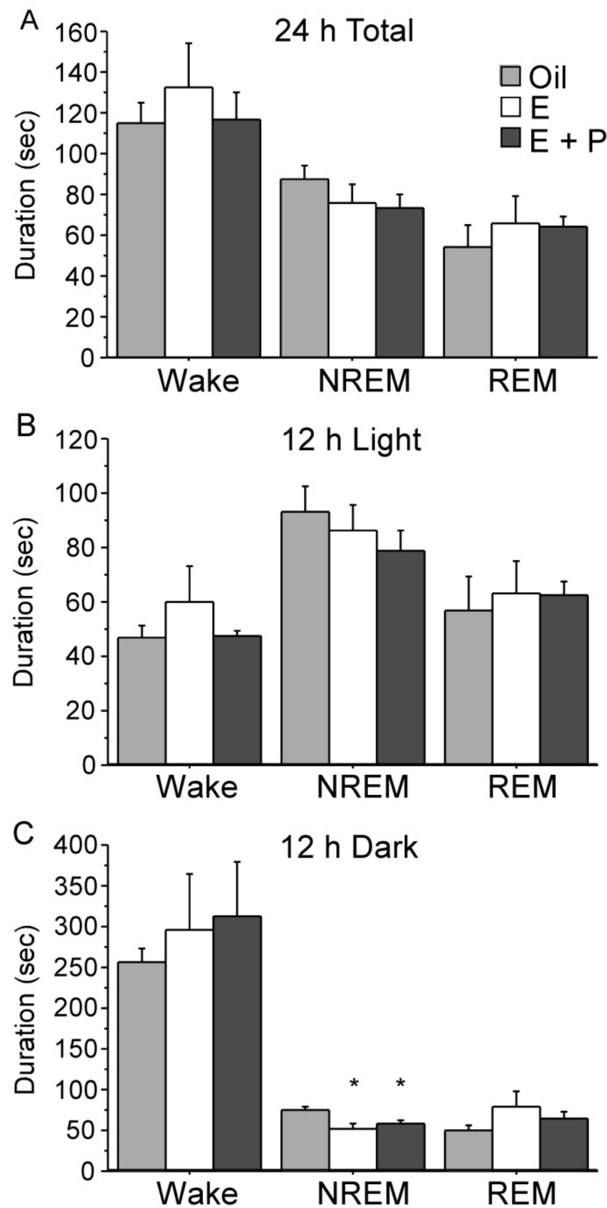


Figure 6. Duration of episodes of Wake, NREMS, REMS during baseline recording for the full 24 h period (A), 12 h light phase (B) and the 12 h dark phase (C) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). Animals treated with E, either alone or in combination with P, displayed decreased duration of NREMS episodes during the dark phase (C). There were no significant differences between groups in the light phase or over the full 24h period. * Different from Oil group; all $P < 0.05$ (Fisher LSD post hoc comparisons).

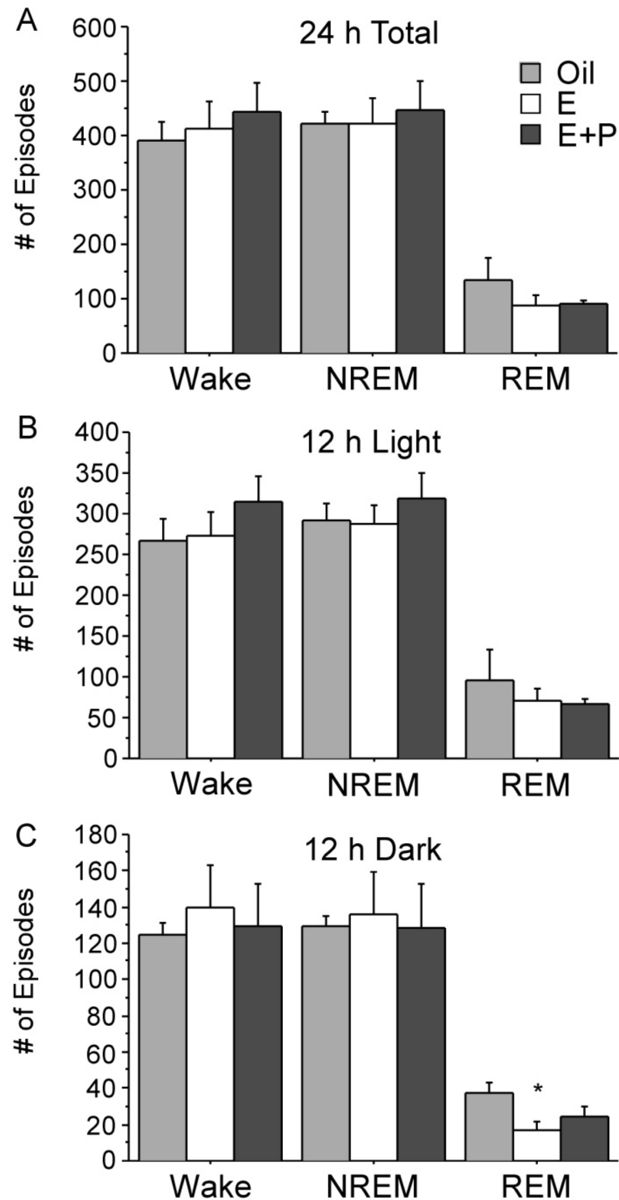


Figure 7. Number of episodes of Wake, NREMS, REMS during baseline recording for the full 24 h period (A), 12 h light phase (B) and the 12 h dark phase (C) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). Animals treated with E displayed a decreased number of REMS episodes during the dark phase (C). There were no significant differences between groups in the light phase or over the full 24h period. * Different from Oil group; all $P < 0.05$ (Fisher LSD post hoc comparisons).

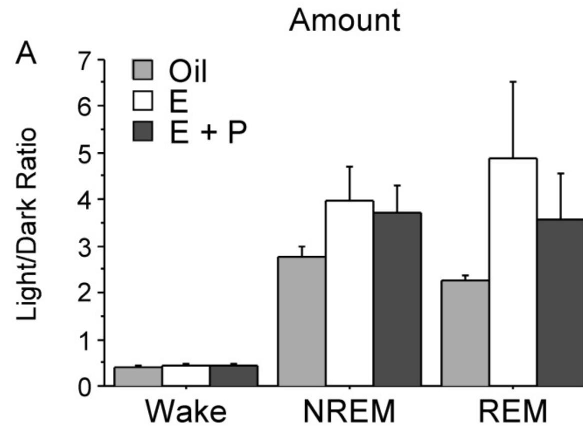


Figure 8. Light/dark ratio for the amount of Wake, NREMS, REMS during baseline recording for the full 24h period in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). The trends of increased light/dark ratios in the E and E+P groups were not significant. All $P < 0.05$ (Fisher LSD post hoc comparisons).

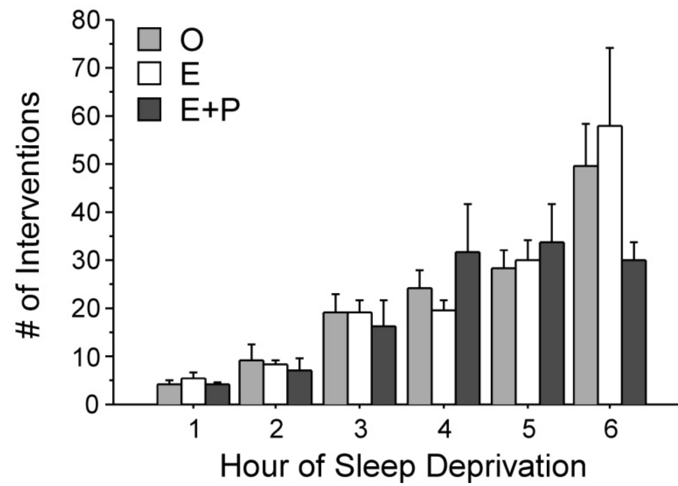
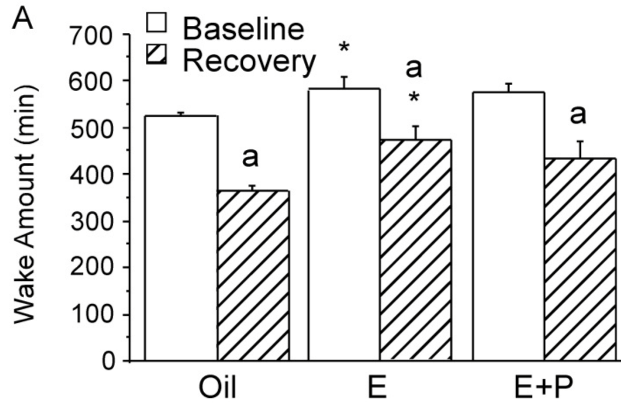
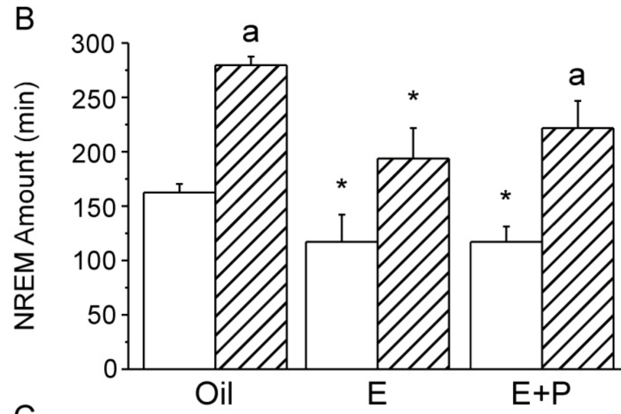
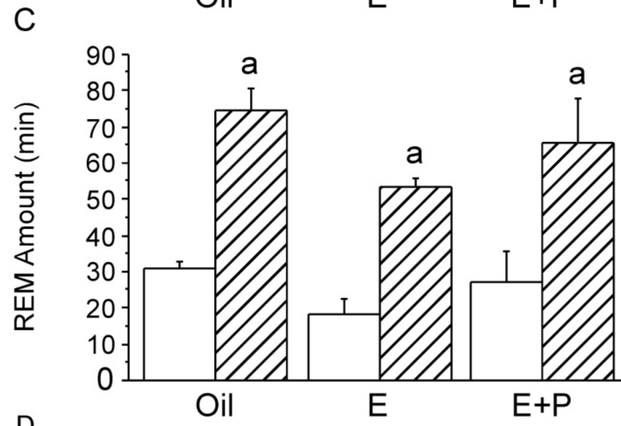


Figure 9. Number of interventions per hour during sleep deprivation. The number of interventions increased across the sleep deprivation period, without any significant variations between groups. Data are shown as mean + SEM for 5 animals per group. (Fisher LSD post hoc comparisons).

Figure 10. Amount of Wake (A), NREMS (B), and REMS (C) during the first 12 h of recovery (dark phase) following sleep deprivation (shaded) and during the dark phase of the baseline recording (white), and the percent change of each sleep/wake state from baseline (D) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). All groups showed decreased amounts of wake (A), increased NREMS (B) and increased REMS (C) during recovery compared to baseline recordings, although the increase in NREMS during recovery compared to baseline did not reach statistical significance in E treated animals. E treated animals showed increased baseline and recovery amounts of wake (A), and decreased baseline and recovery amounts of NREMS (B) compared to the Oil treated group, although they showed the same percent change as Oil treated animals. E+P treated animals displayed a decreased baseline amount of NREMS (B) compared to the Oil treated group. The percent change in amounts of wake, NREMS or REMS between recovery and baseline (D) did not differ between treatment groups. * Different from Oil group, a – different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).


A
Baseline
Recovery
Wake Amount (min)
700
600
500
400
300
200
100
0
*
a
*
a
a
Oil
E
E+P


B
NREM Amount (min)
300
250
200
150
100
50
0
a
*
a
*
*
Oil
E
E+P


C
REM Amount (min)
90
80
70
60
50
40
30
20
10
0
a
a
a
Oil
E
E+P

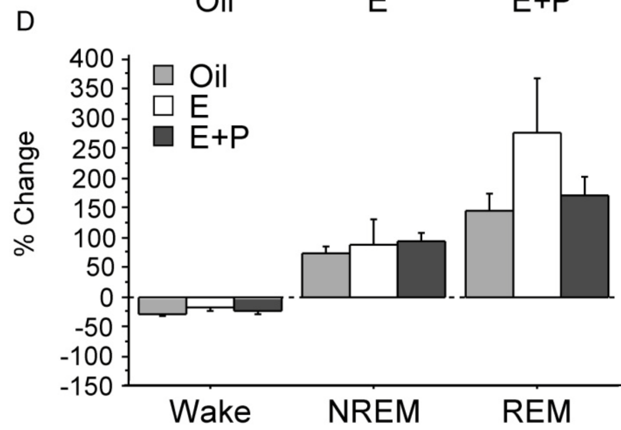
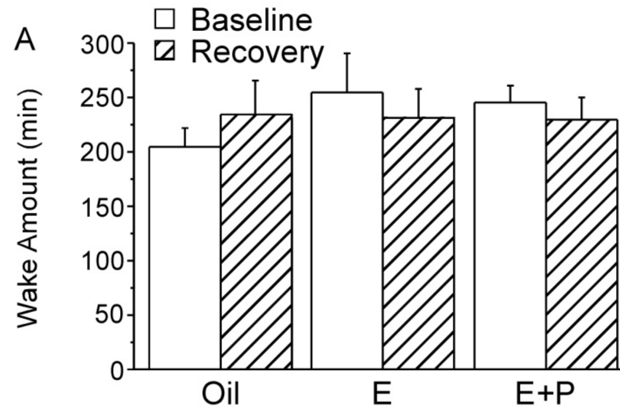
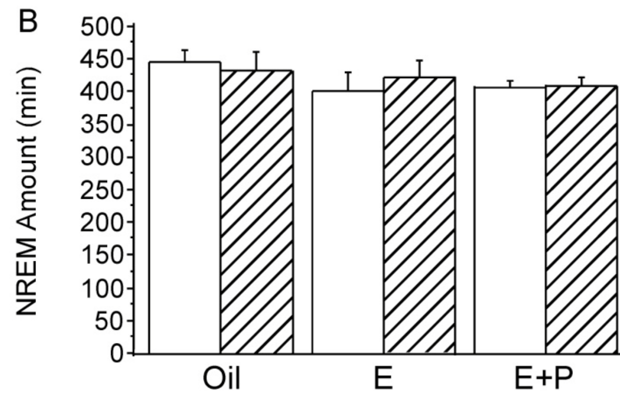
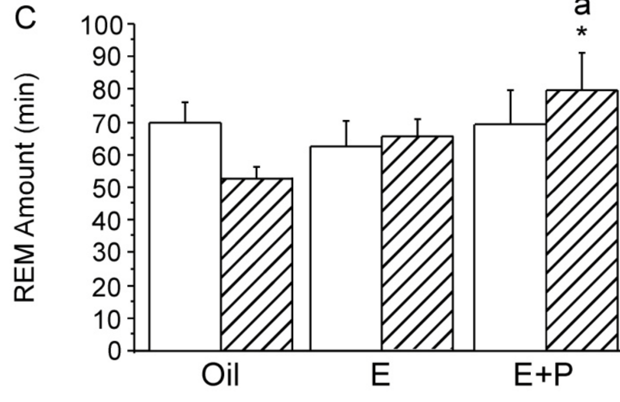
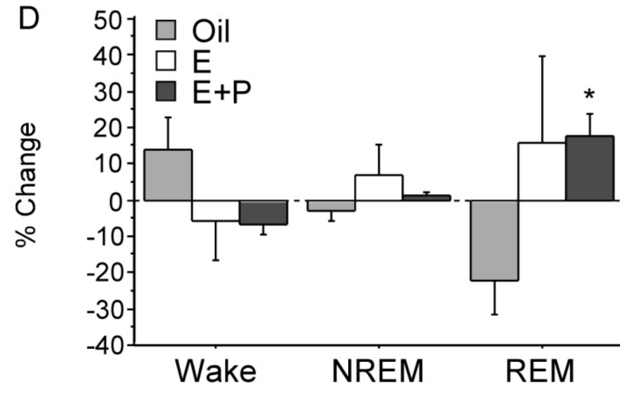

D
% Change
400
350
300
250
200
150
100
50
0
-50
-100
-150
Oil
E
E+P
Wake
NREM
REM

Figure 11. Amount of Wake (A), NREMS (B), REMS (C) during the second 12 h (light phase) of recovery following sleep deprivation (shaded) and during the light phase of the baseline recording (white), and the percent change of each sleep/wake state from baseline (D) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). E+P treated animals displayed increased amounts of REMS (C) during recovery compared to baseline and increased recovery amounts of REMS (C) compared to the Oil treated group. E+P treated animals also showed a greater percent change in REMS amount between baseline and recovery compared to the Oil treated group.. There were no significant differences found between groups or between recovery and baseline within groups in amounts of wake or NREMS in the light phase.* Different from Oil group, a – different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).


A
Baseline
Recovery
Wake Amount (min)
300
250
200
150
100
50
0
Oil
E
E+P


B
NREM Amount (min)
500
450
400
350
300
250
200
150
100
50
0
Oil
E
E+P


C
REM Amount (min)
a
*
100
90
80
70
60
50
40
30
20
10
0
Oil
E
E+P


D
Oil
E
E+P
% Change
*
50
40
30
20
10
0
-10
-20
-30
-40
Wake
NREM
REM

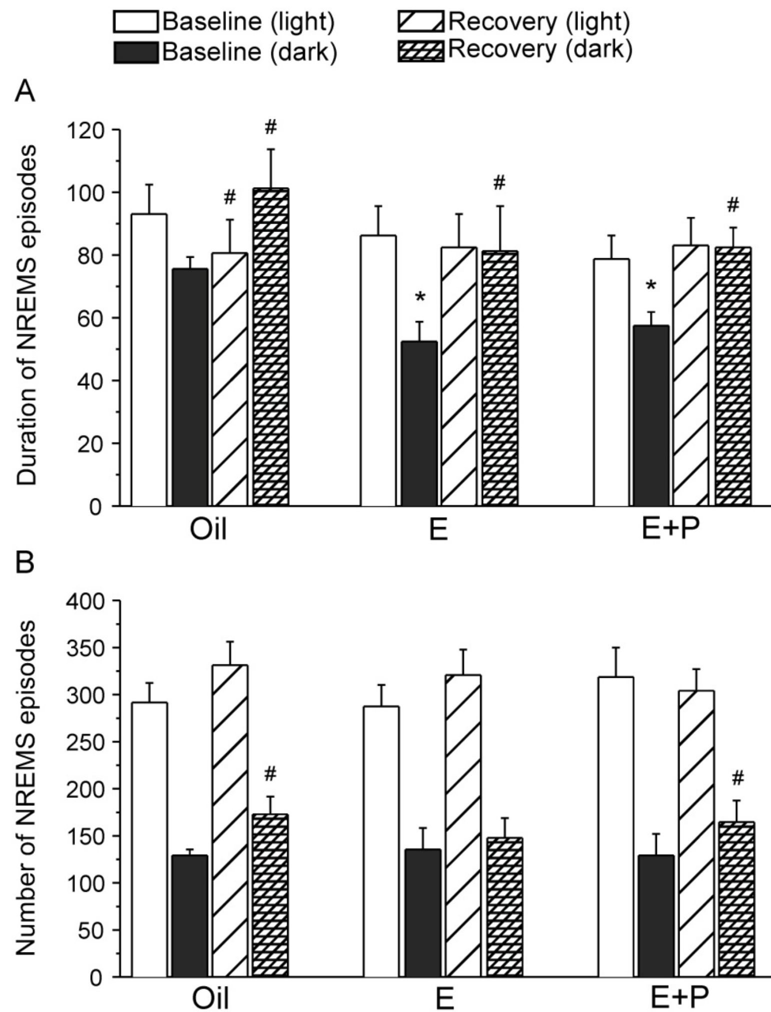


Figure 12. Duration (A) and number (B) of NREMS episodes during baseline and recovery following sleep deprivation, presented separately for the light and dark phases, in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). Oil treated animals showed increased number and duration of NREMS episodes during the dark phase of recovery compared to corresponding baseline period, and an increased duration of NREMS episodes during the light phase of recovery compared to baseline. E treated animals showed decreased duration of NREMS episodes during the dark phase of baseline, compared to Oil, and increased duration of NREMS episodes during recovery dark phase compared to the corresponding baseline period. E+P treated animals showed a decreased duration of NREMS episodes during the dark phase of baseline, compared to Oil, and an increased number and duration of NREMS episodes during the dark phase of recovery compared to corresponding baseline period. * Different from Oil group, # – different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).

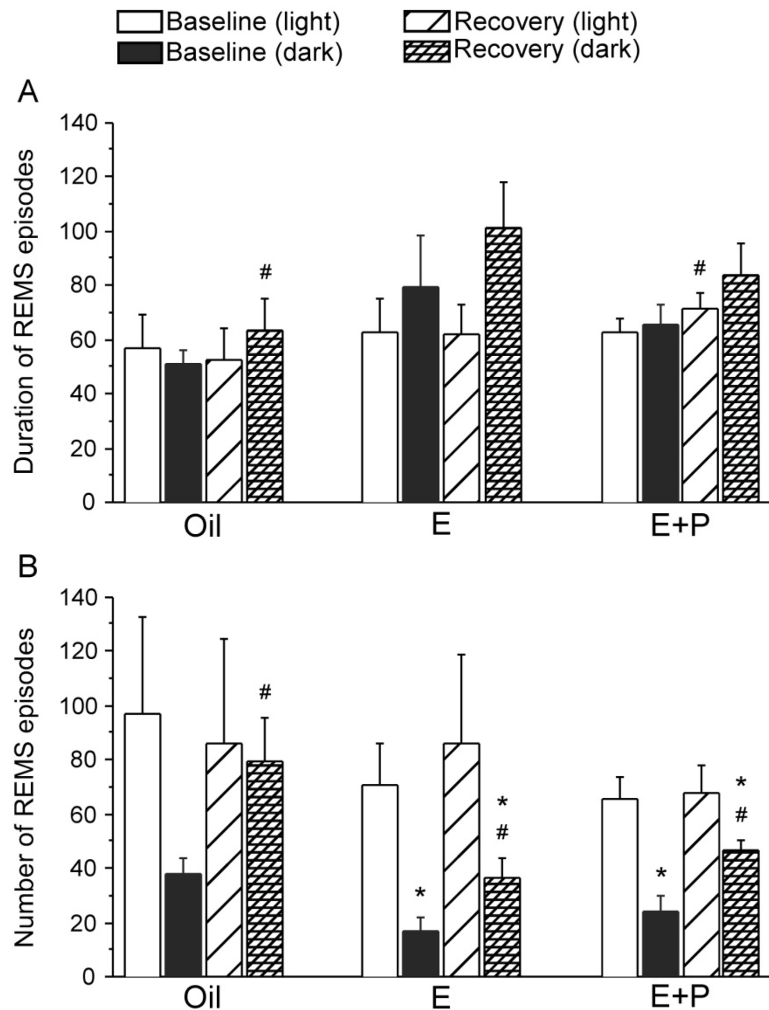


Figure 13. Duration (A) and number (B) of REMS episodes during baseline and recovery following sleep deprivation, presented separately for the light and dark phases, in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). Oil treated animals showed increased number and duration of REMS episodes during the dark phase of recovery compared to corresponding baseline period. E and E+P treated animals showed decreased number of REMS episodes during the baseline dark phase and during the recovery dark phase, compared to Oil, and increased number of REMS episodes during recovery dark phase compared to the corresponding baseline period. E+P treated animals also showed increased duration of REMS in the light phase (second half) of recovery, compared to the corresponding baseline period. * Different from Oil group, # – different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).

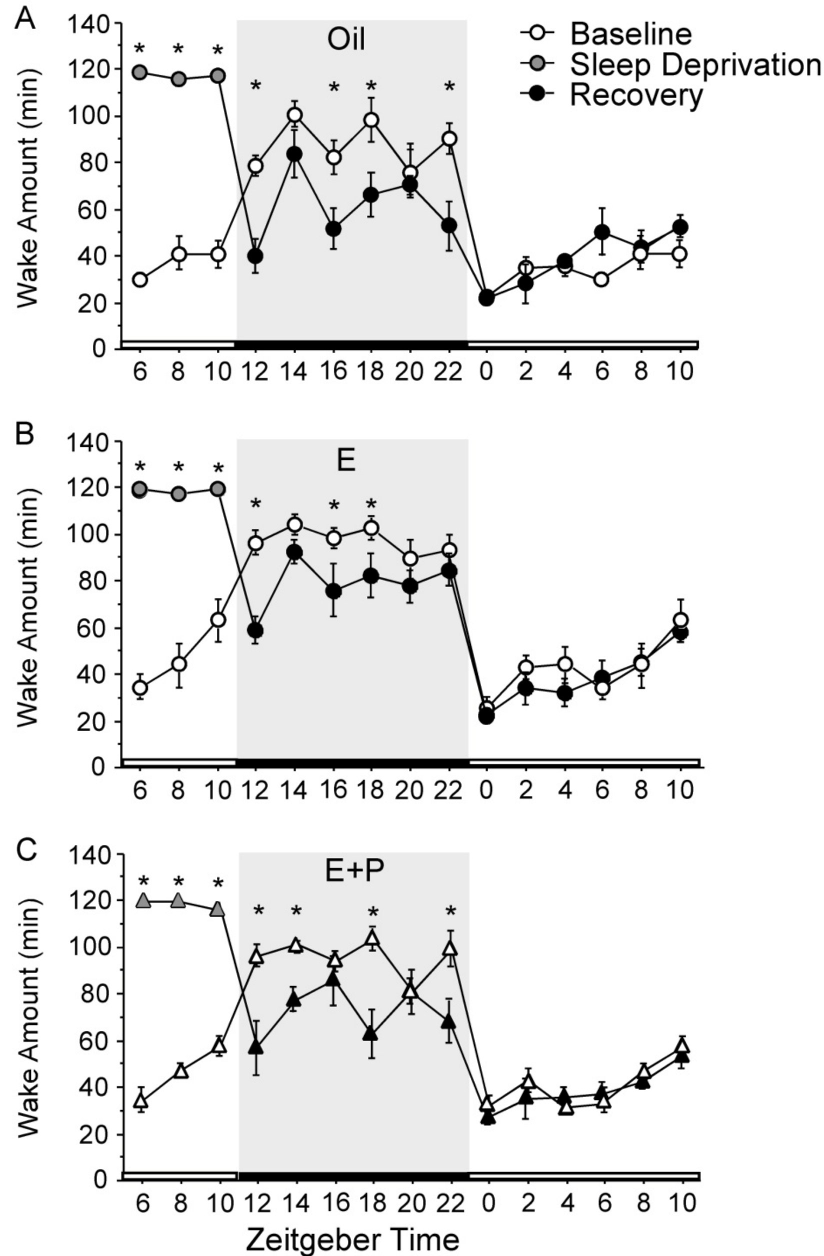


Figure 14. Time course of amount of Wake in 2 h intervals across 24 h baseline recording (white), during 6 h sleep deprivation (grey), and during 24 h recovery period (black) in ovariectomized middle-aged female rats treated with Oil (shown in A), estrogen (E, shown in B), or estrogen & progesterone (E+P, shown in C). The amount of wake was significantly lower during the first 2 h of recovery following sleep deprivation in all treatment groups, then increased over the course of the dark phase, while still remaining lower than baseline values. Wake amounts returned to baseline values at the beginning of and during the subsequent light phase of recovery for all groups. Baseline time points for Zeitgeber time 6, 8, and 10 are displayed twice in order to facilitate comparisons between baseline and recovery periods. * Different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).

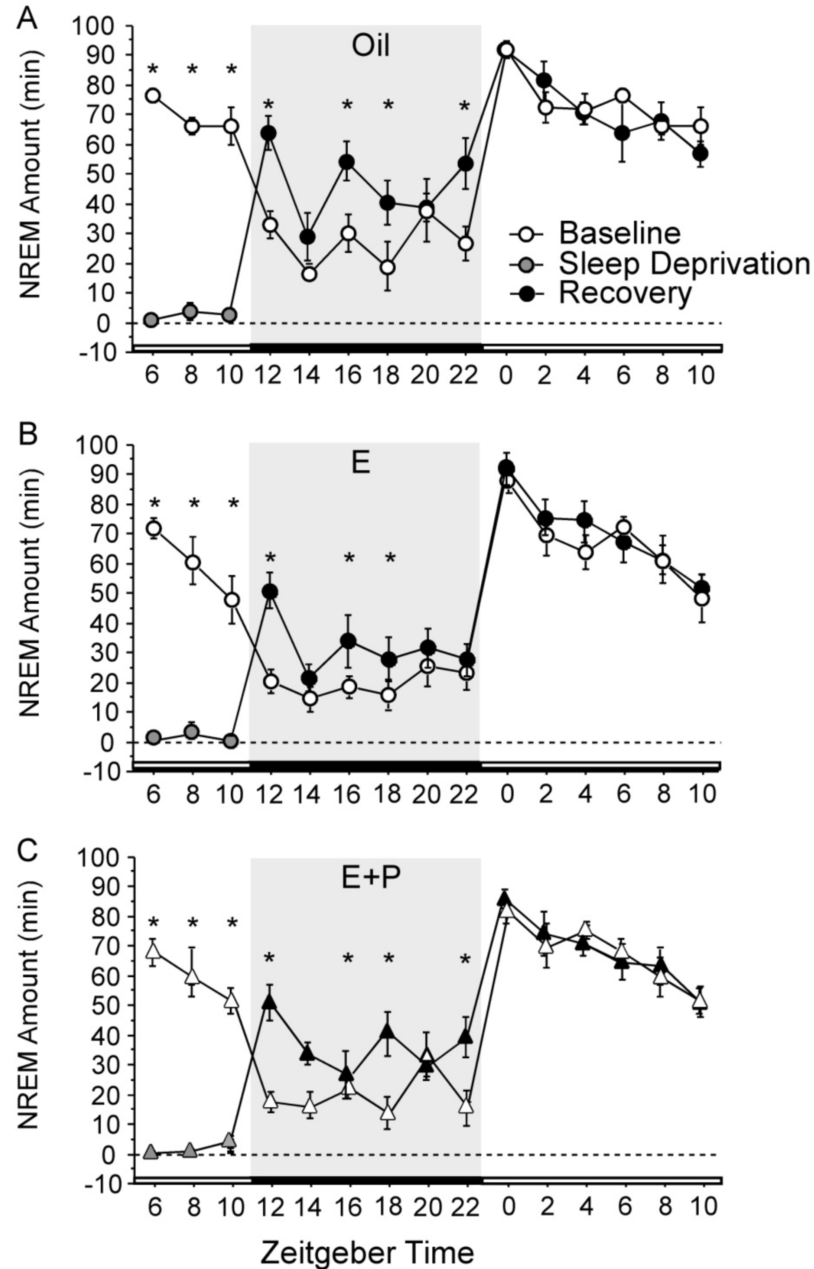


Figure 15. Time course of amount of NREMS in 2 h intervals across 24 h baseline recording (white), during 6 h sleep deprivation (grey), and during 24 h recovery period (black) in ovariectomized middle-aged female rats treated with Oil (shown in A), estrogen (E, shown in B), or estrogen & progesterone (E+P, shown in C). The amount of NREMS was elevated during the first 2h of recovery following sleep deprivation in all treatment groups, then decreased over the course of the dark phase, while still remaining above the baseline values. NREMS amounts returned to baseline values during the subsequent light phase of recovery for all groups. Baseline time points for Zeitgeber time 6, 8, and 10 are displayed twice in order to facilitate comparisons between baseline and recovery periods.* Different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).

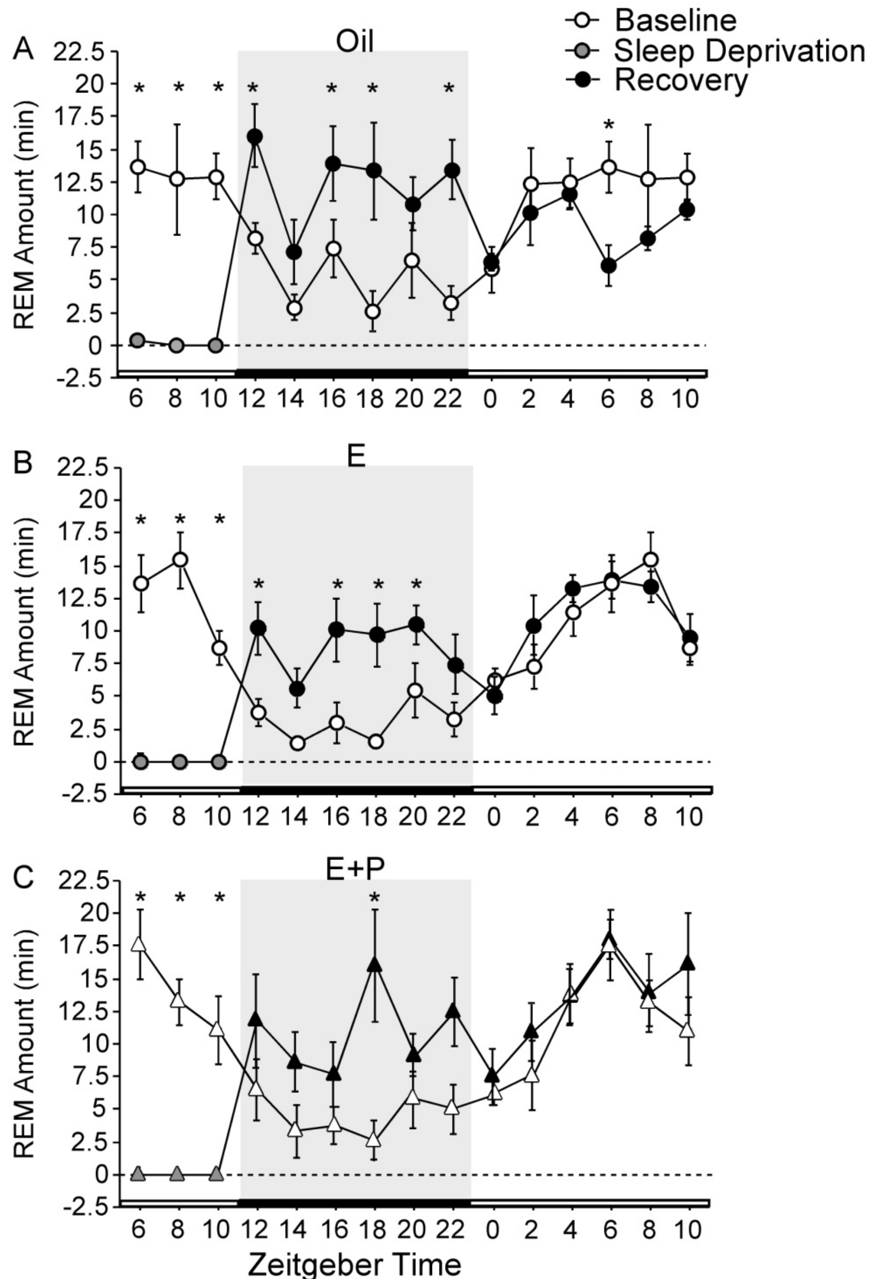


Figure 16. Time course of amount of REMS in 2 h intervals across 24 h baseline recording (white), during 6 h sleep deprivation (grey), and during 24 h recovery period (black) in ovariectomized middle-aged female rats treated with Oil (shown in A), estrogen (E, shown in B), or estrogen & progesterone (E+P, shown in C). The amount of REMS was elevated during the first 2h of recovery following sleep deprivation in all treatment groups, with increases in REMS amount continuing throughout the dark phase. REMS amounts returned to baseline values at the beginning of the subsequent light phase of recovery for all groups, except for oil treated animal who displayed a negative rebound around the middle of the light phase. Baseline time points for Zeitgeber time 6, 8, and 10 are displayed twice in order to facilitate comparisons between

baseline and recovery periods.* Different from corresponding baseline; all P < 0.05 (Fisher LSD post hoc comparisons).

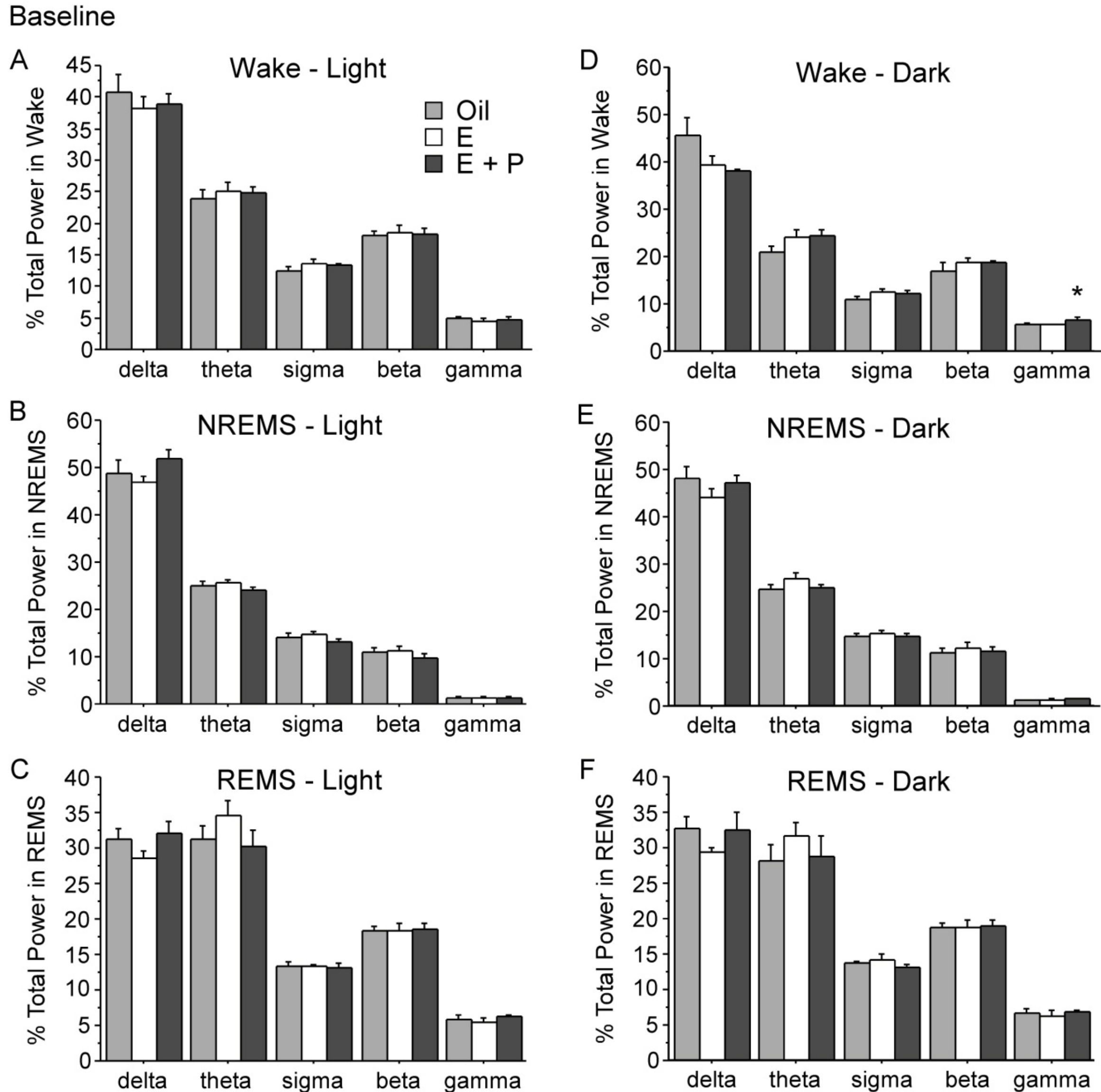


Figure 17. EEG power values in 5 frequency bands in Wake, NREMS, REMS during baseline recording for the 12 h light phase (A, B, C) and the 12 h dark phase (D, E, F) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). EEG spectral power was normalized to the total power (0.5-50 Hz) in each animal. Animals treated with E and P displayed increased gamma power during episodes of wake in the dark phase (D). There were no significant differences found between groups for any frequency band in the light phase. * Different from Oil group; all P < 0.05 (Fisher LSD post hoc comparisons).

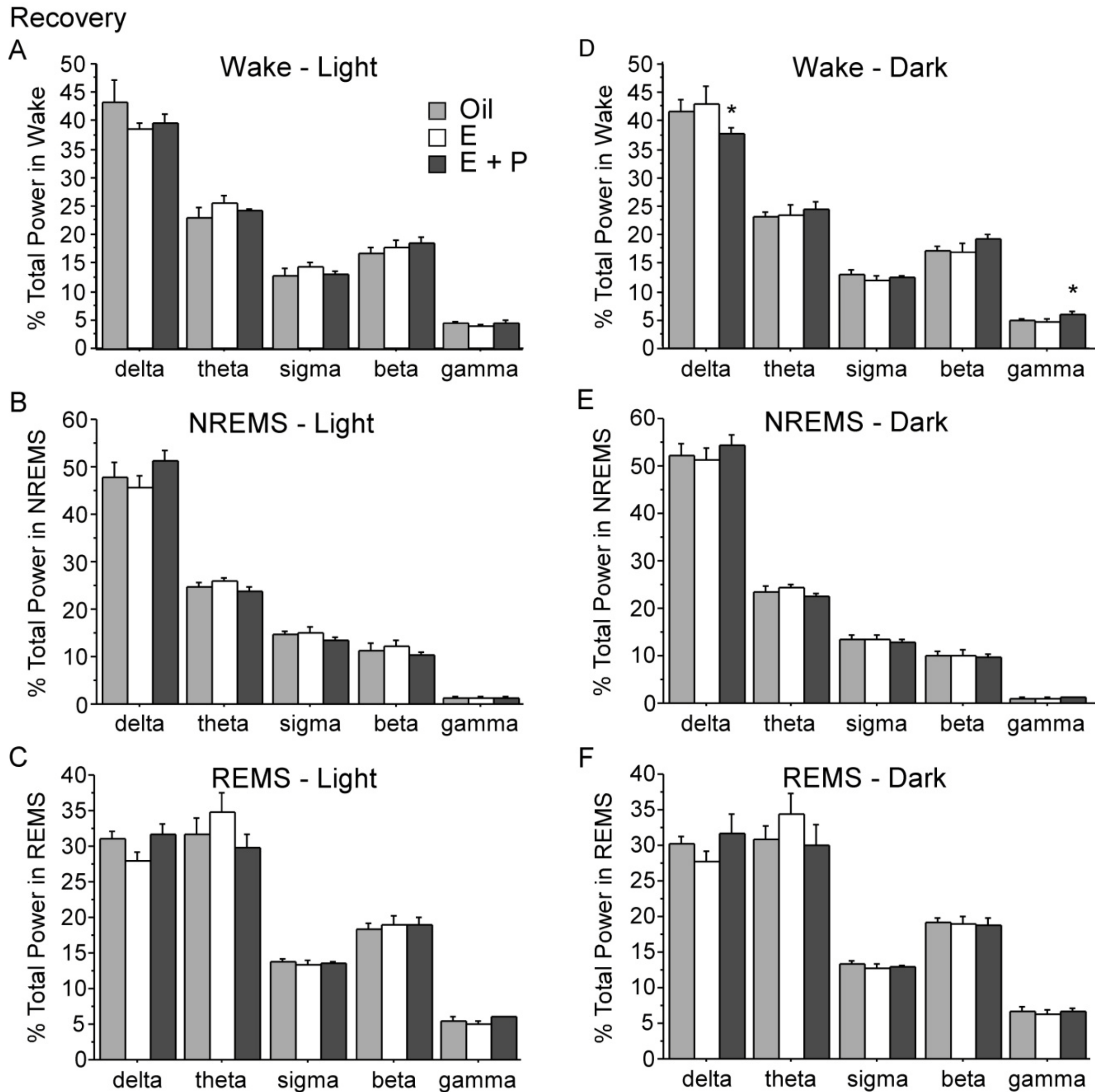
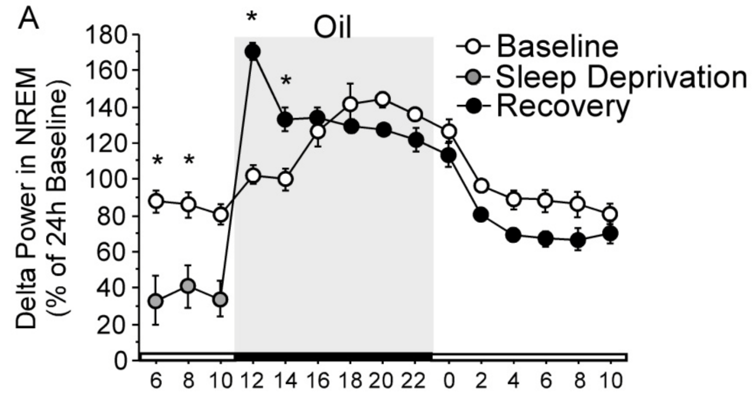
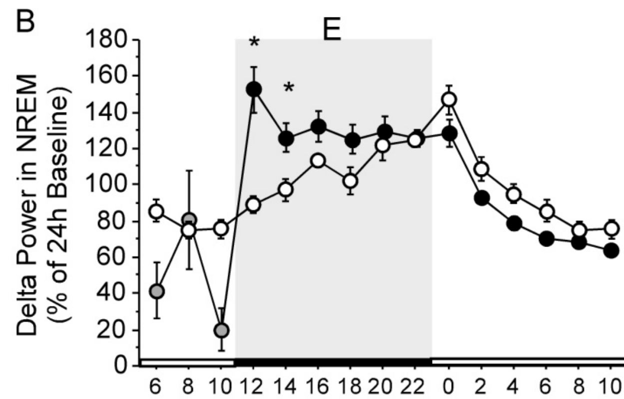
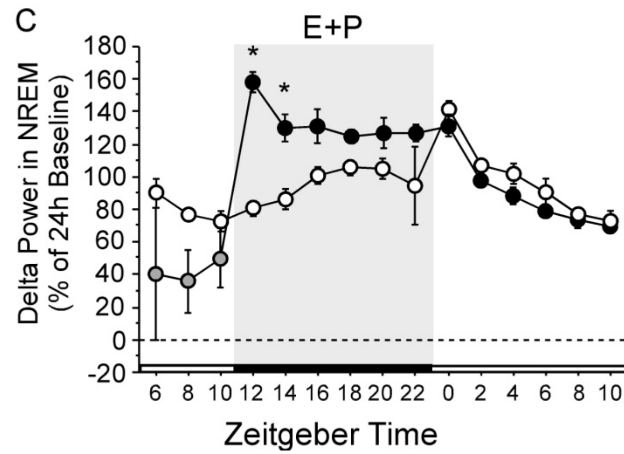


Figure 18. EEG power values in 5 frequency bands in Wake, NREMS, REMS during recovery from sleep deprivation for the 12h light phase (A, B, C) and the 12h dark phase (D, E, F) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). EEG spectral power was normalized to the total power (0.5-50 Hz) in each animal. Animals treated with E and P displayed increased gamma power and decreased delta power during episodes of wake in the dark phase (D). There were no significant differences found between groups for any frequency band in the phases. * Different from Oil group; all P < 0.05 (Fisher LSD post hoc comparisons).

Figure 19. Time course of % delta power during NREMS in 2 h intervals across 24 h baseline recording (white circles), during 6 h sleep deprivation (grey circles), and during 24 h recovery period (black circles) in ovariectomized middle-aged female rats treated with oil, estradiol (E), or estradiol & progesterone (E+P). The delta power during NREMS was elevated significantly for the first 4 h of recovery following sleep deprivation in all treatment groups. Delta power during NREMS tended to remain above the baseline values in E and E+P treated animals for the rest of the dark phase, while Oil treated animals showed a trend of negative rebound. NREMS delta power returned to baseline values during the subsequent light phase of recovery for all groups. A trend of negative rebound was observed in all groups, but was particularly in the Oil group, which showed an earlier and a larger reduction in NREMS delta power during recovery compared to baseline. Baseline time points for Zeitgeber time 6, 8, and 10 are displayed twice in order to facilitate comparisons between baseline and recovery periods.* Different from Oil group, a – different from corresponding baseline; all $P < 0.05$ (Fisher LSD post hoc comparisons).


A
Oil
Baseline
Sleep Deprivation
Recovery
Delta Power in NREM
(% of 24h Baseline)
180
160
140
120
100
80
60
40
20
0
6 8 10 12 14 16 18 20 22 0 2 4 6 8 10
*
*
*
*


B
E
Delta Power in NREM
(% of 24h Baseline)
180
160
140
120
100
80
60
40
20
0
6 8 10 12 14 16 18 20 22 0 2 4 6 8 10
*
*


C
E+P
Delta Power in NREM
(% of 24h Baseline)
180
160
140
120
100
80
60
40
20
0
-20
6 8 10 12 14 16 18 20 22 0 2 4 6 8 10
Zeitgeber Time
*
*

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