

Sex Differences in Susceptibility to Oxidative Injury and Sleepiness From Intermittent Hypoxia

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Study Objectives: Adult male mice exposed to long-term intermittent hypoxia (LTIH), modeling sleep apnea oxygenation patterns, develop nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent residual hypersomnolence and oxidative injury in select brain regions, including wake-active regions. Premenopausal females are less susceptible to selective oxidative brain injuries. We sought to determine whether female mice exposed to LTIH would confer resistance to LTIH-induced wake impairments and oxidative injuries.

Subjects and Setting: Young adult male and female C57Bl/6J mice were studied in a university laboratory.

Interventions: Mice were randomly assigned to either LTIH or sham LTIH for 8 weeks. Total (24-h) wake time and mean sleep latency were measured under 2 conditions: rested and following 6 hours of enforced wakefulness. NADPH oxidase activation, carbonylation, and lipid peroxidation assays were also performed to assess sex differences in oxidative responses to LTIH.

Results: In contrast with the significant LTIH-induced wake impairments

observed in male mice, females following LTIH showed normal wake times and sleep latencies. Female mice revealed less baseline carbonylation and less carbonylation following LTIH but showed robust NADPH oxidase activation and lipid peroxidation. In contrast with the female relative resistance to LTIH sleepiness, female mice showed more-pronounced sleepiness and delta response after enforced wakefulness.

Conclusions: Despite a robust oxidative response to LTIH, age-matched female mice may be protected, at least temporarily, from LTIH wake impairments by lower basal carbonylation. In contrast, females show greater wake impairments after sleep deprivation. We hypothesize sex differences in polysomnographic predictors of sleepiness and residual sleepiness in humans with sleep apnea.

Keywords: Intermittent hypoxia, NREM sleep, peroxynitrite, oxidation

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INTRODUCTION

OBSTRUCTIVE SLEEP APNEA (OSA) WITH DAYTIME HYPERSOMNOLENCE IS PRESENT IN AT LEAST 12 MILLION ADULTS IN THE UNITED STATES.¹ TWO THIRDS of adults with OSA complain of significant sleepiness and/or fatigue at presentation.² Yet, therapies may not fully reverse hypersomnolence.³⁻⁶ Thus, residual sleepiness in adults treated for OSA is a significant health concern.

Adult male mice exposed to hypoxia/reoxygenation patterns similar to those present in patients with severe OSA develop lasting hypersomnolence and objective sleepiness.⁷ These wake impairments are associated with oxidation and nitration in wake-active regions.⁷⁻⁹ Both the oxidative injury and wakefulness impairments are dependent upon nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity.⁹ Thus, the long-term intermittent hypoxia (LTIH)-induced residual wake impairments are a consequence of NADPH oxidase-dependent oxidative injury in selective brain regions. Perinatal LTIH exposure in male

and female rat pups results in wake impairments lasting at least 3 weeks, associated with alterations in dopaminergic signaling.^{10,11} Studies have shown sex differences in dopaminergic receptor and reuptake proteins, yet show no differences of posthypoxia sleep-wake architecture.^{10,11} Whether there are sex differences in wake-impairment effects of LTIH in young adults is not known.

Sex differences have been shown for oxidative brain injuries. In humans, female sex is associated with improved outcomes for both cerebrovascular insults and Parkinson disease.^{12,13} In experimentally induced oxidative brain injuries in mice, premenopausal females confer protection relative to age-matched males or ovariectomized age-matched females.¹⁴ These findings suggest that endogenous estrogen plays an important role in mechanisms of protection from brain oxidative injuries (for review, see reference 15). We hypothesized that premenopausal female mice, relative to age-matched male mice, would show protection from LTIH oxidative injury and wake impairments.

The purpose of the studies presented here was to compare the effects of LTIH exposure in young adult male and female mice on wake impairments and oxidative injury to wake regions in the brain. We first determined whether hypoxia/reoxygenation events, modeling severe OSA hypoxia/reoxygenation, manifest as residual hypersomnolence and shortened sleep latencies in females, as we have found in males. In the present work, we have found that females appear to be resistant to LTIH wake impairments, both hypersomnolence and sleepiness. To gain further insight into potential mechanisms of sleepiness in women with OSA, we next measured sleep-latency responses in male and female mice exposed to short-term sleep loss. In contrast with the lack of LTIH effect on wake function in female mice, female mice showed greater sleepiness after short-term sleep loss and more robust slow-wave (delta) activity across recovery. We then

Disclosure Statement

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determined whether LTIH induces NADPH oxidase activation and oxidative injury in select wake-active brain regions in female mice, as has been shown in male mice. We have found less baseline oxidative injury in female mice, such that, despite LTIH increases in carbonylation in this sex, levels remained lower than those of male mice under sham-LTIH conditions. This work suggests that females are susceptible to the physiologic perturbances of OSA, but, because of a lower oxidative injury at baseline, are protected, at least temporarily, from the intermittent-hypoxemia effects. At the same time, female mice experience greater objective sleepiness in response to sleep disruption, as compared with age-matched male mice. Elucidation of sex differences in OSA wake impairments will advance our understanding of clinically significant polysomnographic indexes for each of the 2 sexes.

METHODS

Animals

Eight- to 10-week-old male and female littermate C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were studied. Methods and study protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Protocols conformed to the revised National Institutes of Health Office of Laboratory Animal Welfare Policy. Food and water were provided ad libitum. Mice were confirmed pathogen free at the time of behavioral studies.

LTIH Protocol

Detailed descriptions of the LTIH protocol were recently published.^{9,16} An automated nitrogen/oxygen delivery profile system (Oxycycler model A84XOV; Biospherix, Redfield, NY) delivered 100% nitrogen to the chambers to produce rapid brief reductions in the fraction of inspired oxygen (FiO_2) within the chamber from 21% to 10% for 5 seconds every 90 seconds. Arterial oxyhemoglobin saturation in males and females at a constant FiO_2 of 21% was 95% to 98% and at an FiO_2 of 10% was 83% to 86%. There were no sex differences in oxyhemoglobin saturation. Sham LTIH, with ambient FiO_2 fluctuations from 21% to 19% every 90 seconds, held arterial oxyhemoglobin values constant between 96% and 98%. Both conditions were produced for 10 hours of the lights-on period for a total of 8 weeks. Prior to exposures, during exposures, and across recovery and sleep studies, lights were turned on at 7:00 AM and turned off at 7:00 PM. Humidity, ambient CO_2 , and environmental temperature were held constant.

Sleep-Wake, Multiple Sleep Latency, and Enforced-Wakefulness Protocols

Following 8 weeks of LTIH or sham LTIH exposures, mice were returned to normoxic conditions and randomly assigned to sleep or biochemical assays. Mice randomly assigned to sleep studies underwent surgical implantation of electroencephalographic and electromyographic electrodes after 1 week in normoxia using previously described methods.¹⁷ Mice were connected to recording cables in individual cages, and sleep recordings were begun 7 days postoperatively. Baseline sleep was recorded for 6 days. On baseline recording day 6, a murine Multiple Sleep Latency Test¹⁸ was performed across 4 nap opportunities between 2:00 PM and 4:00 PM to measure baseline sleep propensity. On recording day 7, enforced wakefulness was performed (using gentle air

puffs and placement of cotton for nest building) with verification of electroencephalogram tracings for 6 hours of the light period (8:00 AM to 2:00 PM), followed by a second sleep-latency test and then recovery sleep was recorded for 12 hours. The behavioral-state acquisition and analysis program used for these studies was previously described,¹⁹ with modifications and behavioral-state parameters.^{17,18} Primary variables were total wake time per 24 hours, total non-rapid eye movement (NREM) sleep time per 24 hours, rapid eye movement (REM) sleep time per 24 hours, length of average wake bout, and average sleep latency before and after short-term sleep loss.

NADPH Oxidase Subunits Rac 1 and p67^{phox}

Translocation of NADPH oxidase cytosolic subunits to the membrane indicates NADPH oxidase activation, as measured by Western.²⁰ Cytosolic and membrane/organelle fractions in WT mice exposed to LTIH or sham LTIH ($n=5$ per condition) were separated using 100,000 G \times 60 minutes.²⁰ Polyclonal rabbit antimouse rac 1 and p67^{phox} (1:500, Upstate, Lake Placid, NY) were added to homogenates and bound by a horseradish peroxidase conjugated secondary anti-IgG (1:15,000, 12-349, Upstate) for detection with chemiluminescence (SuperSignal Ultra, Pierce, Rockford, IL). Images were analyzed with National Institutes of Health Image Analysis, using band densities for statistical comparisons (rac: membrane fraction:cytosolic/organelle fraction) and p67^{phox}: membrane density, as the cytosolic/organelle fraction density was too weak to measure). Preliminary trials showed weaker immunoreactivity 2 weeks into recovery. Thus, these measures were made on the last day of LTIH or sham LTIH.

Measurement of Carbonyl Proteins and F₂ Isoprostanes

For measurement of protein carbonylation, macrodissections of the locus coeruleus in the dorsal pons were procured for protein purification. Purified protein (20 μg) was run on enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit (Zentec PC Test, Zenith Technology, Dunedin, New Zealand) as recently described.^{8,9} Measurement of isoprostane, d₄-8,12-iso-iPF_{2a}-VI, (iPF_{2a}-VI) was performed 2 weeks into recovery, as previously described,^{21,22} using whole forebrains in mice following conditions of LTIH and sham intermittent in male and female mice ($n = 5/\text{group}$). Total lipid was extracted with ice-cold Folch solution.²¹ Lipids were then subjected to base hydrolysis with aqueous 15% KOH and were then incubated at 45°C for 1 hour for measurement of total iPF_{2a}-VI using ion chemical ionization gas chromatography-mass spectrometry assay, as previously described.²²

Statistical Analysis

Values reported represent mean \pm SEM. To assess LTIH and sex differences in sleep-wake parameters, 2-way analysis of variance (ANOVA) was used with the dependent variables total wake time and wake bout length (Bonferroni corrected for the three variables) and the independent variables intermittent-hypoxia condition and sex. To assess sleep loss and intermittent-hypoxia effects on sleep latencies in female mice, 2-way ANOVA was used with sleep latency as the dependent variable and sleep loss or rested conditions and LTIH or sham LTIH conditions as the 2 independent variables. To determine whether female

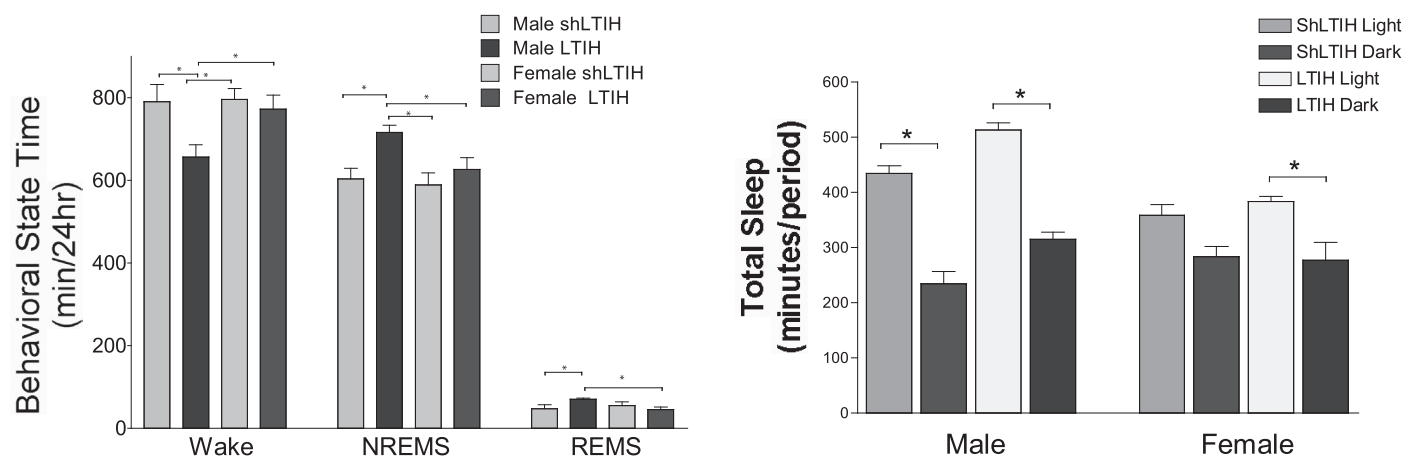


Figure 1—Sex-dependent effects of long-term intermittent hypoxia (LTIH) on sleep-wake times. A. Time spent in each of 3 behavioral states: wakefulness (Wake), non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep were averaged for male mice exposed to sham LTIH (ShLTIH, $n=15$) and LTIH ($n=15$) and for females for the same conditions ($n=13$ and 14 , respectively). Error bars signify standard error (SE). *Denotes sex and LTIH with $p < .05$. Female mice confer resistance to LTIH-induced wake impairments observed in males. B. Sex and LTIH effects on total sleep time in light and dark periods. Total NREM and REM sleep time per 12-h lights-on period (Light) and lights-off period (Dark) expressed as average for sex and intermittent-hypoxia condition \pm SE. *Denotes Bonferroni corrected statistical differences in light versus dark total sleep times.

mice developed increased isoprostanes with LTIH exposure, 1-way ANOVA comparing LTIH and sham LTIH was performed. Similarly, to determine whether LTIH activated NADPH oxidase, 1-way ANOVA was used comparing LTIH and sham LTIH membrane-band densities. The latter was done independently for the 2 wake sites (basal forebrain and locus coeruleus) in females. To ascertain sex-specific gene-response differences, 2-way ANOVA was used with copy number for each of the 4 genes as a primary variable (Bonferroni corrected), using independent variables of sex and LTIH condition. The null hypothesis was rejected for probabilities $< .05$.

RESULTS

Female Mice Confer Resistance to LTIH Reduced Wake Times

Wake, NREM sleep, and REM sleep times per 24 hours were measured in female mice following conditions of sham LTIH ($n = 13$) and LTIH ($n = 14$) and compared with values obtained in male mice following conditions of sham LTIH ($n = 15$) and LTIH ($n = 15$). Results are summarized in Figure 1. Across conditions of sham LTIH, there were no sex differences in wake time (females wake time = 795 ± 26 minutes and males = 770 ± 25 minutes, $t = 0.8$, NS). Comparing sham LTIH to LTIH, male mice showed a large reduction in wake time (173 minutes less wake, $t = 5.9$, $p < .001$). In contrast, female mice showed no effect of LTIH on total wake time (wake time: sham LTIH 795 ± 26 minutes vs LTIH 767 ± 33 minutes, $t = 0.9$, NS). Thus, wake time in males following LTIH exposure was significantly less than wake time in females after LTIH exposure (difference, 170 minutes more wake time in females after LTIH, $t = 6.1$, $p < .001$).

LTIH effects on NREM sleep directly opposed the LTIH effects on wakefulness. In males, LTIH increased NREM sleep time by 130 minutes, compared with sham LTIH, ($t = 4.4$, $p < .001$). In females, LTIH had no effect on NREM sleep time. The difference of 37 more minutes after LTIH was not significant, ($t = 1.3$). There was a large increase in REM sleep after LTIH in males

(42 minutes, $t = 2.8$, $p < .05$) but no change in REM sleep time in females across LTIH conditions (mean difference 10 minutes, $t = 0.3$, NS).

There were also sex effects on sleep architecture for the light and dark periods of the 24-hour cycle, as summarized in Figure 1B. Male mice showed a larger diurnal variation for sleep in the lights on period (200 minutes more sleep in lights on, ($t = 7.46$, $p < .001$). In contrast, females had less light-to-dark difference (100 minutes more sleep in lights on, $t = 3.7$, $p < .01$). Consistent with previous findings, males showed remarkably parallel increases across the entire 24-hour period.⁷ There was a 77-minute increase in LTIH-exposed mice, relative to sham-LTIH mice ($t = 2.9$, $p < .05$) in the lights-on period and a 77-minute increase in the dark period in LTIH mice relative to sham LTIH mice ($t = 2.9$, $p < .05$). In contrast, females showed no difference in either the light (difference, 44-minute increase, $t = 1.6$, NS) or the dark period (28-minute decrease, $t = 1.0$, NS).

Sleep-Latency Responses to LTIH and Sleep Loss are Sex Specific

Murine multiple sleep latency testing was performed on males and females following conditions of sham LTIH and LTIH, and comparisons were drawn between males and females across the 2 LTIH conditions.

Baseline Sleep Latencies for Sham LTIH

A summary of results is depicted in Figure 2. The baseline (rested) mean sleep latency for male and female mice following sham-LTIH conditions did not differ. The mean latency for males was 13.6 ± 0.7 minutes ($n = 11$), and, for females, the latency was 12.9 ± 1.0 minutes ($n = 12$, $t = 0.5$, NS).

Baseline Sleep Latencies for LTIH Across Sexes

Male mice exposed to LTIH had an average latency of 9.6 ± 1.1 minutes ($n = 12$), and female mice following LTIH had a latency of 13.1 ± 1.2 minutes, for a difference of -3.5 minutes ($t = 2.7$, $p < .05$).

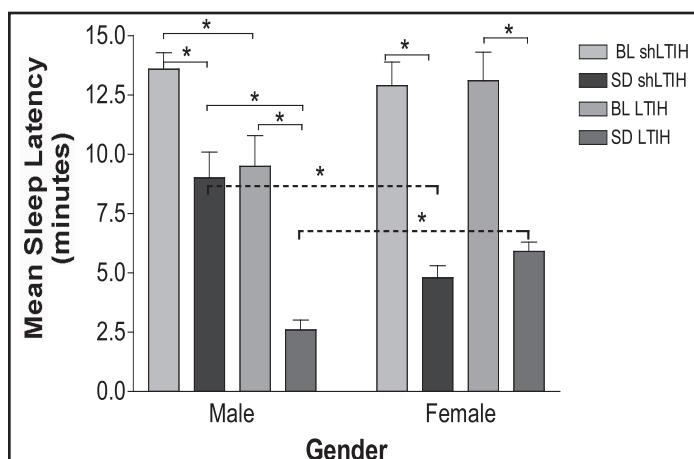


Figure 2—Sex-specific effects on sleep-latency responses to both long-term intermittent hypoxia (LTIH) and enforced wakefulness. Multiple sleep latency testing was performed on male and female mice under the following conditions: baseline (uninterrupted rest in the lights-on period) sham LTIH (BL ShLTIH), baseline LTIH (BL) and for the 2 LTIH conditions following 6 hours of enforced wakefulness in the lights-on period (SD ShLTIH and SD LTIH). Sample sizes were 11–12 mice per sex per LTIH/BL vs sleep-deprived conditions. Error bars denote SE. Thin solid bars represent statistical differences within LTIH or baseline vs sleep deprivation, and dashed bold lines signify sex differences. *Represents statistical differences of $p < .05$.

Effects of LTIH on Baseline Latency for Each Sex

There was an effect of intermittent-hypoxemia condition on sleep latency in male mice (LTIH vs sham LTIH difference was -4.0 minutes, $t = 3.1$, $p < .05$). In contrast, there was no intermittent-hypoxemia effect on sleep latencies in female mice (difference $+0.2$ minutes, $t = 0.3$, NS). In summary, there were no sex differences in baseline sleep latencies for sham LTIH, and LTIH did not affect the sleep latency in female mice, as it did affect sleep latency in male mice.

Both males and females had significant effects of enforced sleep loss on sleep latencies for conditions of sham LTIH and LTIH.

Effects of Sleep Deprivation on Sham LTIH Sleep Latency for Each Sex

Short-term sleep loss in males exposed to sham LTIH shortened the sleep latency by 4.7 minutes ($t = 3.5$, $p < .01$). In contrast, short-term sleep loss in females exposed to sham LTIH shortened the sleep latency by 8 minutes ($t = 6.6$, $p < .001$).

Effects of Sleep Deprivation on LTIH Sleep Latency for Each Sex

Short-term sleep loss in male mice exposed to LTIH shortened the sleep latency by 7 minutes ($t = 5.4$, $p < .001$). A similar effect of sleep deprivation was seen in females exposed to LTIH, with the sleep latency shortened by 7 minutes, ($t = 5.8$, $p < .001$).

Effects of Sleep Deprivation on Sham-LTIH Sleep Latency Across Sexes

Following short-term sleep deprivation, the sham LTIH-exposed male mice had an average sleep latency of 8.9 ± 1.1 minutes ($n = 11$), whereas, after short-term sleep deprivation, sham LTIH-exposed female mice had an average sleep latency of 4.8 ± 0.5 minutes (difference $+4.1$ minutes, $t = 3.2$, $p < .05$).

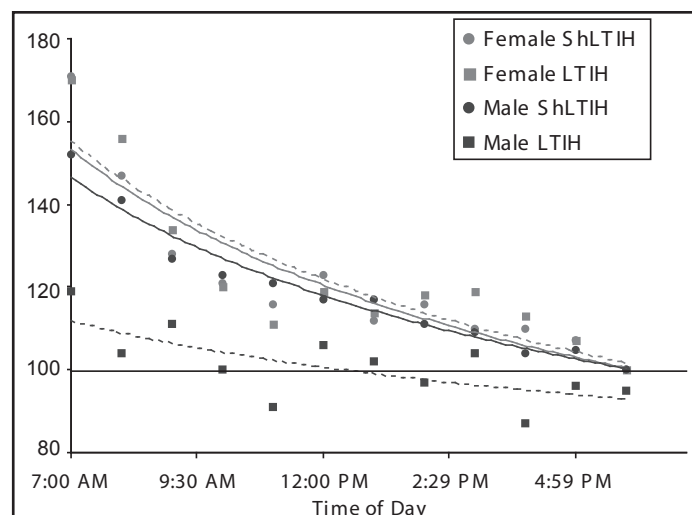


Figure 3—Sex-specific effects of long-term intermittent hypoxia (LTIH) on relative delta power across the lights-on period. Delta power was measured for each 10-second epoch across the lights-on period to provide an average delta power for each hour of the day. The values here are expressed relative to the end of the lights-on period as a percentage of the 6:00 PM value. In male and female mice following conditions of sham LTIH (male ShLTIH, black circle, solid black line; female ShLTIH, gray circle, solid gray line), delta power is high at the beginning of the lights-on period and sharply declines over the ensuing hours. Male mice following LTIH conditions show less of a change in delta power across the rest-predominant period (male LTIH, black square, dashed black line). In contrast, females following LTIH show a robust delta decline, as seen for sham LTIH conditions (female LTIH, gray square, dashed gray line).

Effects of Sleep Deprivation on LTIH Sleep Latency Across Sexes

Following LTIH and short-term sleep deprivation, sleep latencies were shorter in males than females. After short-term sleep loss, LTIH males had an average sleep latency of 2.6 ± 0.4 minutes ($n = 12$). In contrast, LTIH females after short-term sleep loss had an average sleep latency of 5.9 ± 0.7 minutes, for a difference of $+3.2$ minutes ($t = 2.7$, $p < .05$), unchanged from the sham-LTIH sleep-deprivation baseline. In summary, in contrast with the LTIH effects that were more pronounced in males, the sleep-loss effects on sleep latency were greater in female mice for sham-LTIH conditions. However the combination of sleep loss and LTIH resulted in a shorter sleep latency in males.

Female Mice Confer Resistance to LTIH-Induced Disruption in Slow-Wave Activity

Delta decline across the lights-on period was determined for 9 female mice exposed to LTIH and 9 female mice exposed to sham LTIH, and data are presented in Figure 3. There were no sex differences in delta decline for sham-LTIH conditions across the lights-on period in rested mice. In contrast with LTIH effects reported in male mice,^{7,8} female mice showed no difference in maximal delta power percentage at the onset of the lights-on period (sham LTIH: $171\% \pm 7\%$ and LTIH: $179\% \pm 15\%$, $t = 0.47$, NS). Similarly, in female mice, the slope of delta decline did not differ across LTIH conditions (sham LTIH: -4.4 ± 0.4 and LTIH: -5.1 ± 0.9 , $t = 0.74$, NS).

Delta activity in response to 6 hours of enforced wakefulness was observed in 9 female and 9 male mice exposed to sham LTIH and 9 female and 9 male mice exposed to LTIH. Data are not shown but are presented below. A large increase in delta activity

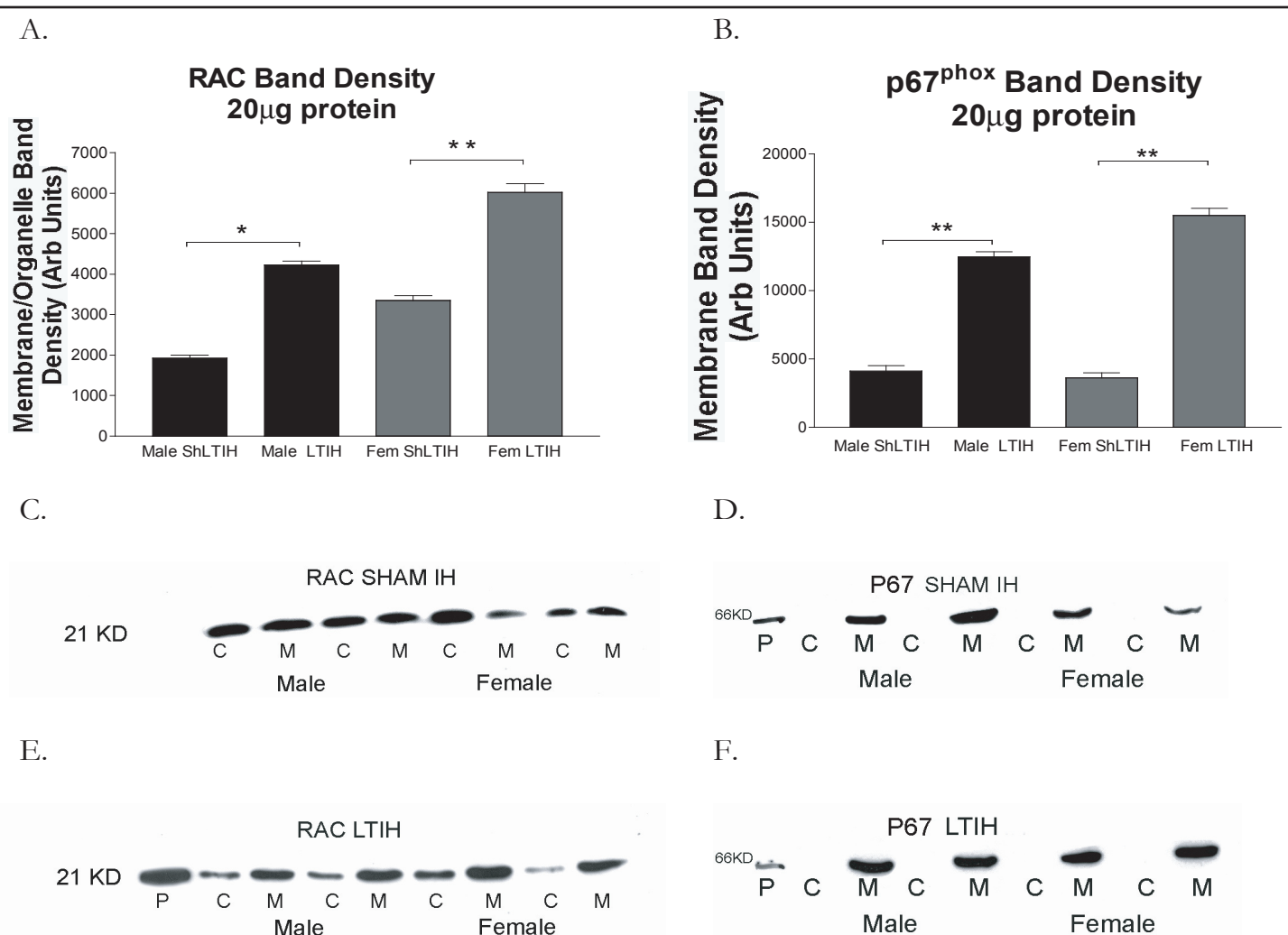


Figure 4—Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in dorsal pons in response to long-term intermittent hypoxia (LTIH). As an index of NADPH oxidase activation membrane translocation on Westerns was measured for 2 of the 5 NADPH oxidase subunits: rac 1 and p67^{phox} in dorsal pons macropunches. Comparisons were drawn for male and female wild-type mice following conditions of sham LTIH (Sh-LTIH) and LTIH. Values presented average densities \pm SE for 20-μg protein loaded. Panel A shows membrane:cytosol and organelle fractions for rac protein. * $p < .05$ and ** $p < .01$. Panel B shows p67^{phox} membrane densities for 20-μg protein loaded. ** $p < .001$. Panels C and E are representative Westerns for rac in which P is the positive control at 21 KD, C represents the cytosolic/organelle fraction, and M represents the membrane fraction. Overall, under conditions of LTIH, the membrane:cytosol/organelle band-density ratio increases, suggesting NADPH oxidase activation. Panels D and F are representative Westerns for p67^{phox} protein. The P in this case denotes 66 KD position, and cytosol/organelle fractions are not detected in the dorsal pons for this weaker antibody.

was observed in female mice for the first hour of recovery sleep for both groups of female mice (sham LTIH mice: $265\% \pm 23\%$ and LTIH mice: $285\% \pm 34\%$, $t = 0.83$, NS). In contrast, male mice exposed to sham LTIH had a smaller increase after 6 hours of enforced wakefulness, relative to female mice exposed to sham LTIH ($169\% \pm 32\%$, $t = 4.3$, $p < .01$). Differences in delta decline were even larger across sexes for mice exposed to LTIH. Males exposed to LTIH showed a minimal increase in delta activity for the first hour of recovery sleep ($138\% \pm 28\%$, $t = 6.5$, $p < .001$). In summary, female mice showed larger increases in relative delta power after short-term sleep loss, and this increased delta response in females was not affected by LTIH, as it was in males.

No Sex Differences in NADPH Oxidase Activation in Response to LTIH

Densities of membrane-bound p67 and rac 1 subunits were used as an index measure of NADPH oxidase activation.²⁰ For NADPH oxidase subunit rac 1, there was no sex effect in sham-LTIH mice

(Figure 4A) (Bonferroni multiple comparison $t = 1.7$, NS). Both sexes showed increased rac 1 translocation to the membrane in response to LTIH (females $t = 3.3$, $p < .01$; males $t = 2.8$, $p < .05$). There was no difference between LTIH male or female rac 1 densities ($t = 2.2$, NS). Similar findings were observed with NADPH oxidase subunit p67 (Figure 4B), although the magnitude of LTIH response for each sex was greater. There were no differences in baseline p67 membrane densities ($t = 0.02$, NS). Females and males each showed robust translocation in response to LTIH ($t = 7.5$, $p < .001$ and $t = 6.0$, $p < .01$, respectively). There were no sex differences in LTIH membrane densities ($t = 1.5$, $p < .05$).

Sex-Specific Differences in Baseline Carbonylated Proteins in Wake-Active Brain Regions and Reduced Carbonylation in LTIH Females

There were large sex and LTIH effects on the concentration of carbonyl protein in the locus coeruleus ($F = 18$, $p < .0001$) (Figure 5A). Carbonyl content in female sham-LTIH mice was

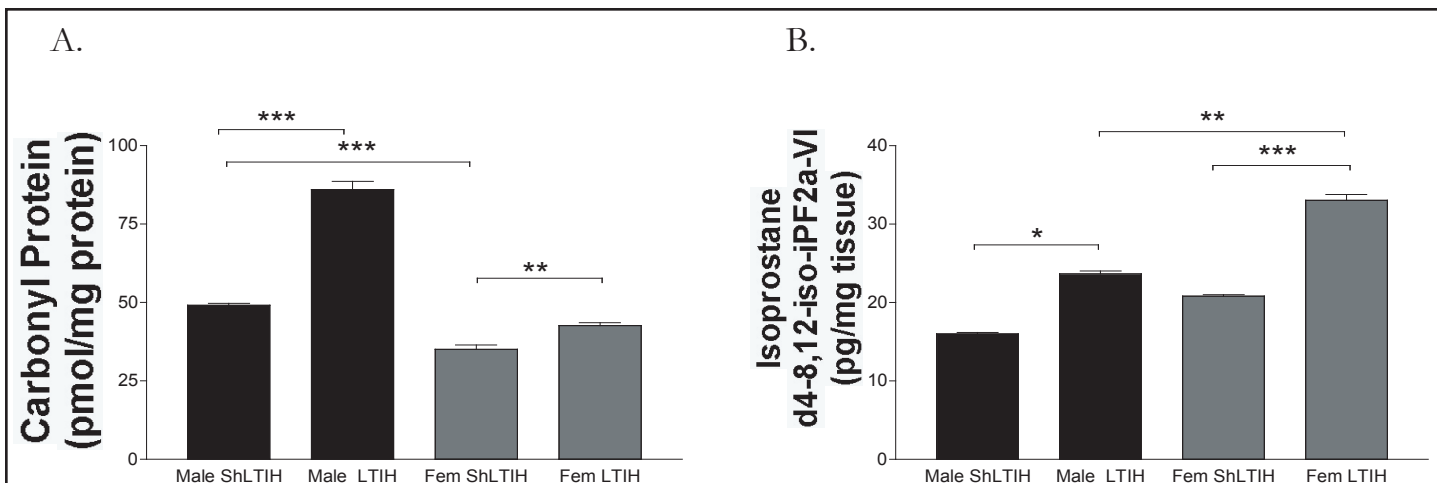


Figure 5—Carbonyl protein and lipid peroxidation in male and female mice exposed to long-term intermittent hypoxia (LTIH). A. Carbonyl content was measured with enzyme-linked immunosorbent assay in dorsal pontine macropunches in male mice under conditions of sham LTIH (male ShLTIH) and LTIH (male LTIH) and under the same conditions in age-matched female mice (female ShLTIH and female LTIH) for sample sizes of 5 mice per sex per LTIH condition. Carbonyl content (carbonyl protein/20-mg protein aliquot) is significantly increased in male mice relative to female mice for the sham-LTIH condition. Following LTIH, carbonyl is increased in both sexes but is far higher in males. * $p < .05$; ** $p < .01$ and *** $p < .001$. B. Homogenized tissue samples were collected for measurement of isoprostane, 8,12-iso-iPF₂-VI, from the cortex in female and male mice following 8 weeks of exposure to either LTIH or sham LTIH. Using an internal standard, levels were assayed by negative-ion chemical ionization gas chromatography and mass spectrometry. * $p < .05$; ** $p < .01$; *** $p < .001$.

lower than in males ($t = 4.6$, $p < .001$). LTIH increased carbonyl content in females ($t = 4.8$, $p < .001$) and also in males ($t = 2.7$, $p < .05$). However, carbonyl content was significantly lower in female mice exposed to LTIH than in males under the same exposure ($t = 3.3$, $p < .01$) and, in fact, was close to the level of carbonylation in males under sham conditions ($t = 0.7$, NS).

Females Show Greater Lipid Peroxidation in Response to LTIH

Both males and females showed LTIH increases in isoprostane d₄-8,12-iso-iPF_{2a}-VI (iPF_{2a}-VI) levels (Figure 5B). There was no sex effect on sham LTIH iPF_{2a}-VI, ($t = 1.6$, NS). Females and males showed increases in iPF_{2a}-VI in response to LTIH (females: $t = 4.2$, $p < .001$ and males: $t = 2.6$, $p < .05$). However, the increase in females was larger, such that the iPF_{2a}-VI levels in LTIH females were significantly higher than the levels in LTIH-exposed males, ($t = 3.2$, $p < .01$).

DISCUSSION

We have obtained direct evidence for important sex differences in neurobehavioral and neurochemical responses to LTIH, modeling the hypoxia/reoxygenation events in OSA, and we have also observed significant sex differences in the response to short-term sleep deprivation. In contrast with the robust wake impairments observed in young adult male mice following LTIH, age-matched female mice confer resistance to LTIH-induced shortened total wakefulness time across a 24-hour period and increased objective sleepiness, as measured by the murine multiple sleep latency test. Previously, we have shown that oxidative injury contributes substantially to the LTIH-induced wake impairments in male mice.^{10,11} In the present work, we have obtained evidence that female mice are susceptible to LTIH-induced NADPH oxidase activation and both lipid peroxidation and carbonylation injuries. In light of the strong proinflammatory response in female mice, we suspect that the resistance to neurobehavioral sequelae is short lived. The proinflammatory response is likely to increase oxidative neural

injury so that, ultimately, the female mice are expected to have sufficient carbonylation injury.²⁰ A unique advantage observed in the females was lower baseline carbonylation. Thus, whether the females have a more robust antioxidant capacity at baseline and throughout LTIH should next be determined.

The LTIH Model of Hypoxia/Reoxygenation Events of Sleep Apnea

LTIH was achieved by way of fluctuating the levels of nitrogen and oxygen in the chambers in which mice resided. This model provides the capability to explore long-term effects of hypoxia/reoxygenation events in an intact in vivo preparation. A very similar protocol has been implemented in young rats and mice to show LTIH-induced cognitive dysfunction and oxidative injury in the cortex, hippocampus, and wake-active brain regions.^{9-11,23-25} Findings with this model are consistent with brain injury identified in persons with OSA.^{26,27} By design, the LTIH model excludes hypercapnic, arousal, and upper-airway perturbances. Further studies are needed to determine if less-severe LTIH can induce these injuries and if hypercapnia, upper-airway responses, and arousals result in either additive or synergistic injuries.

Sex Differences in Wake Function Following LTIH

Several lines of evidence support the concept that young adult female mice are less susceptible to wake impairments induced by LTIH, as in our protocol. First, the study was adequately powered to detect 10% or greater changes for both wake time and sleep latency in females, based on sham-LTIH sleep-latency group average and variability. Indeed, we were able to show in this study with the same mice that female mice are more susceptible to sleepiness following 6 hours of enforced wakefulness in the lights-on period at the same circadian time point when LTIH effects were measured. Moreover, female mice showed more pronounced slow-wave activity responses than male mice in recovery sleep following short-term sleep loss, and this response is not disturbed by LTIH in the females, as it is in males. Collectively, the present

work identifies significant sex differences in young adult mice in responses to both LTIH and sleep loss. Sex differences in susceptibility to sleep loss and intermittent hypoxia raise the possibility that neurobehavioral consequences of OSA in humans may vary with sex. Future studies should examine whether the sex changes are related to menopause.

Previously, we showed that LTIH-induced wake impairments in male mice are a consequence of NADPH oxidase-dependent oxidative injury to wake-active regions.⁹⁻¹¹ We were surprised to see comparable NADPH oxidase activation in females. Westerns presently require macropunches of tissue; thus, the samples used in our assays include both glia and neurons. We have shown NADPH oxidase in wake-active neurons in the locus coeruleus and lateral basal forebrain. It is possible that females show a robust glial NADPH oxidase response to LTIH but less NADPH oxidase activation and injury in wake-active neurons. Immunohistochemistry and in situ hybridization studies may help determine whether the NADPH oxidase response in the 2 sexes occurs primarily in neurons or microglia. In light of the lower carbonylation in females at baseline and in response to LTIH, we suspect that young adult females are somehow protected from basal carbonylation, so that by the time the female mice completed 8 weeks of LTIH, they had not yet attained a critical level of oxidative damage to wake-active neurons to render them dysfunctional. We would predict then that longer exposures or more severe exposures are required for LTIH to result in residual hypersomnolence in young adult females.

The sex-dependent effects on baseline oxidation in wake-active regions may be related to antioxidant effects of estrogen. Estrogen has been shown to have multiple beneficial effects on redox status, including enhancing superoxide dismutase activity²⁸⁻³⁰ and attenuating TNF- α and NF- κ B responses in the brain.^{31,32}

Carbonylation, rather than lipid peroxidation, predicts susceptibility to LTIH-induced wake impairments.

Many oxidative changes occur in the brain in response to LTIH, including nitration, lipid peroxidation, and oxidation.^{7-9,16,23-25} What remains unclear is which of these redox responses contributes to the behavioral impairments observed with LTIH. Lipid peroxidation and carbonylation are believed to result in irreversible changes.³³ Consistent with previous studies, males and females showed LTIH increases in both lipid peroxidation and carbonylation. Females showed larger increases in isoprostane levels than males in response to LTIH and had higher absolute levels than males exposed to LTIH. In contrast, females had lower absolute carbonylation levels than males for both baseline and LTIH conditions. Carbonylation is a marker of cumulative oxidative injury.³³ Thus, females, at least in wake-active regions, show less baseline oxidative injury. Lower carbonylation in females at baseline might be attributed to activation of estrogen receptors, known to increase transcription of both glutathione peroxidase and superoxide dismutase.^{28,29} Future studies should examine baseline antioxidant activity in wake-active regions of male and female mice at baseline and the protective roles both estrogen and progesterone play in responses to LTIH.

Sex Differences in the Homeostatic Response to Short-Term Sleep Loss

In the present study, we found direct complimentary evidence for sex differences in sleep homeostasis. In healthy young indi-

viduals, homeostasis is best characterized by relative change in delta activity in NREM sleep.³⁴⁻³⁶ Female mice showed larger increases in delta activity during recovery NREM sleep. Similar sex differences in sleep-loss responses have been shown for young adult humans.³⁷ Females show higher delta response to sleep loss in humans.³⁷ The second finding in the present study supporting increased homeostatic response to sleep loss in females is that the female mice showed larger reductions in mean sleep latency (objective sleepiness) after 6 hours of enforced wakefulness, confirmed polysomnographically. Thus, we have established sex differences in young adult mice in the homeostatic responses to sleep disruption, with differences in both delta response and sleep-latency response. In addition, we extend these findings to show that females show greater resistance to LTIH injury to the homeostatic mechanisms. We believe these sex differences will provide a valuable tool with which to delineate mechanisms of impaired homeostasis from hypoxia/reoxygenation events of sleep apnea.

Implications for Humans with OSA

Female mice showed greater sleepiness and a more robust homeostatic response to short-term sleep loss than age-matched male mice, while demonstrating resistance to wake impairments following hypoxia/reoxygenation events, modeling severe OSA. In light of these findings, we hypothesize that the polysomnographic findings more predictive of daytime wake impairments in young adult women would be sleep fragmentation and sleep disruption. Thus, upper airways resistance syndrome through sleep fragmentation may have profound effects on wake function in young adult women. While severe OSA is rare in premenopausal women, more women present to the sleep clinics with snoring, sleepiness, and fatigue who have high arousal indexes, without significant apnea-hypopnea events. These individuals respond symptomatically to continuous positive airway pressure (CPAP), but, because of the low apnea-hypopnea index (requiring 4% desaturations), these individuals do not qualify for CPAP reimbursement. It is hoped that the above-described animal studies will help justify clinical studies in young adult women looking at objective sleepiness and quality-of-life responses to CPAP for treatment of respiratory-related sleep fragmentation. Ideally, the polysomnographic indexes to define clinically significant OSA should be designed for each sex.

In contrast with the importance of sleep fragmentation in women, parameters characterizing hypoxia/reoxygenation events may provide greater predictive value in men. We suspect that this sex effect depends upon estrogen bioavailability, and studies are planned to determine the relative roles of estrogen and progesterone in this sex difference and if the sex effect is lost in women following menopause. It is hoped that this work in animal models begins a dialog to assess the clinical significance of sleep and oxygenation polysomnographic parameters, relative to age and sex.

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REFERENCES

1. Young T, Palta M, Dempsey J, Skatrud J, Weber S, and Badr S.

- The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* 1993;328:1230-5.
2. Chervin RD. Sleepiness, fatigue, tiredness, and lack of energy in obstructive sleep apnea. *Chest* 2000;118:372-9.
3. Engleman HM, Martin SE, Deary IJ, Douglas NJ. Effect of continuous positive airway pressure treatment on daytime function in sleep apnoea/hypopnoea syndrome. *Lancet* 1994; 343:572-5.
4. Douglas NJ, Engleman HM. Effects of CPAP on vigilance and related functions in patients with the sleep apnea/hypopnea syndrome. *Sleep* 2000;23:S147-9.
5. Heinzer R, Gaudreau H, Decary A, Sforza E, Petit D, Morisson F, Montplaisir J. Slow-wave activity in sleep apnea patients before and after continuous positive airway pressure treatment: contribution to daytime sleepiness. *Chest* 2001;119:1807-13.
6. Patel SR, White DP, Malhotra A, Stanchina ML, Ayas NT. Continuous positive airway pressure therapy for treating sleepiness in a diverse population with obstructive sleep apnea: results of a meta-analysis. *Arch Intern Med* 2003;163:565-71.
7. Veasey SC, Davis C, Zhan G, et al. Long-term intermittent hypoxia in mice: protracted hypersomnolence with oxidative injury to sleep-wake brain regions. *Sleep* 2004;27:194-201.
8. Zhan G, Fenik P, Pratico D, Veasey SC. Inducible nitric oxide synthase in long-term intermittent hypoxia: hypersomnolence and brain injury. *Am J Respir Crit Care Med* 2005;171:1414-20.
9. Zhan G, Serrano F, Fenik P, et al. NADPH Oxidase Mediates Hypersomnolence and Brain Oxidative Injury in a Murine Model of Sleep Apnea. *Am J Respir Crit Care Med* 2005;In Press.
10. Decker MJ, Hue GE, Caudle WM, Miller GW, Keating GL, Rye DB. Episodic neonatal hypoxia evokes executive dysfunction and regionally specific alterations in markers of dopamine signaling. *Neuroscience* 2003;117:417-25.
11. Decker MJ, Jones KA, Solomon IG, Keating GL, Rye DB. Reduced extracellular dopamine and increased responsiveness to novelty: neurochemical and behavioral sequelae of intermittent hypoxia. *Sleep* 2005;28:169-76.
12. Dick P, Sherif C, Sabeti S, Amighi J, Minar E, Schillinger M. Gender differences in outcome of conservatively treated patients with asymptomatic high grade carotid stenosis. *Stroke* 2005;36:1178-83.
13. Lyons KE, Hubble JP, Troster AI, Pahwa R, Koller WC. Gender differences in Parkinson's disease. *Clin Neuropharmacol* 1998;21:118-21.
14. Tamas A, Lubics A, Szalontay L, Lengvari I, Reglodi D. Age and gender differences in behavioral and morphological outcome after 6-hydroxydopamine-induced lesion of the substantia nigra in rats. *Behav Brain Res* 2005;158:221-9.
15. Toran-Allerand CD. Estrogen and the brain: beyond ER- α , ER- β , and 1 β -estradiol. *Ann N Y Acad Sci* 2005;1052:136-44.
16. Veasey SC, Zhan G, Fenik P, Pratico D. Long-term intermittent hypoxia: reduced excitatory hypoglossal nerve output. *Am J Respir Crit Care Med* 2004;170:665-72.
17. Veasey SC, Valladares O, Fenik P, et al. An automated system for recording and analysis of sleep in mice. *Sleep* 2000;23:1025-40.
18. Veasey SC, Hsu Y-J, Thayer P, Fenik P. Murine multiple sleep latency test: phenotyping sleep propensity in mice. *Sleep* 2004;27:388-93.
19. Benington JH. Sleep homeostasis and the function of sleep. *Sleep* 2000;23:959-66.
20. Wu DC, Teismann P, Tieu K, et al. NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Proc Natl Acad Sci USA* 2003;100:6145-50.
21. Pratico D, Uryu K, Leight S, Trojanowski JQ, Lee VM. Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *J. Neurosci* 2001;21:4183-7.
22. Uryu K, Laurer H, McIntosh T, et al. Repetitive mild brain trauma accelerates A β deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. *J Neurosci* 2002;22:446-54.
23. Gozal D, Daniel JM, Dohanich GP. Behavioral and anatomical correlates of chronic episodic hypoxia during sleep in the rat. *J Neurosci* 2001;21:2442-50.
24. Row BW, Liu R, Wei X, Kheirandish L, Gozal D. Intermittent hypoxia is associated with oxidative stress and spatial learning deficits in the rat. *Am J Respir Crit Care Med* 2003;167:1540-7.
25. Row, BW, Kheirandish L, Li RC, et al. Platelet-activating factor receptor-deficient mice are protected from experimental sleep apnea-induced learning deficits. *J Neurochem* 2004;89:189-96.
26. Macey PM, Henderson LA, Macey KE, et al. Brain morphology associated with obstructive sleep apnea. *Am J Resp Crit Care Med* 2002;166:1382-7.
27. Morrell MJ, McRobbie DW, Quest RA, Cummin AR, Ghiassi R, Corfield DR. Changes in brain morphology associated with obstructive sleep apnea. *Sleep Med* 2003;4:451-4.
28. Xu Y, Armstrong SJ, Arenas IA, Pehowich DJ, Davidge ST. Cardioprotection by chronic estrogen or superoxide dismutase mimetic treatment in the aged female rat. *Am J Physiol Heart Circ Physiol* 2004;287:H165-71.
29. Huang A, Kaley G. Gender-specific regulation of cardiovascular function: estrogen as key player. *Microcirculation* 2004;11:9-38.
30. Florian M, Freiman A, Magder S. Treatment with 17-beta-estradiol reduces superoxide production in aorta of ovariectomized rats. *Steroids* 2004;69:779-87.
31. Liao SL, Chen WY, Chen CJ. Estrogen attenuates tumor necrosis factor- α expression to provide ischemic neuroprotection in female rats. *Neurosci Lett* 2002;330:159-62.
32. Wen Y, Yang S, Liu R, Perez E, Yi KD, and Koulen P. Estrogen attenuates nuclear factor-kappa B activation induced by transient cerebral ischemia. *Brain Res* 2004;1008:147-54.
33. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2001; 82:47-95.
34. Achermann P, Dijk DJ, Brunner DP, Borbély AA. A model of human sleep homeostasis based on EEG slow-wave activity: quantitative comparison of data and simulations. *Brain Res Bull* 1993;31:97-113.
35. Dijk DJ, Brunner DP, Beersma DG, Borbély AA. Electroencephalogram power density and slow wave sleep as a function of prior waking and circadian phase. *Sleep* 1990;13:430-40.
36. Franken P, Chollet D, Tafti M. The homeostatic regulation of sleep need is under genetic control. *J Neurosci* 2001;21:2610-21.
37. Fukuda N, Fukuda, Honma H, et al. Gender difference of slow wave sleep in middle aged and elderly subjects. *Psychiatr Clin Neurosci* 1999;53:151-3.
38. Corsi-Cabrera M, Sanchez AI, del-Rio-Portilla Y, Villanueva Y, Perez-Garci E. Effect of 38 h of total sleep deprivation on the waking EEG in women: sex differences. *Int J Psychophysiol* 2003;50:213-24.