Influence of Gonadal Hormones on the Behavioral Effects of Intermittent Hypoxia in Mice

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Running Header: Sex Differences in Intermittent Hypoxia

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ABSTRACT

Obstructive sleep apnea (OSA) is characterized by repetitive upper airway obstruction resulting in cyclic intermittent hypoxia (IH) during sleep in affected individuals. OSA occurs more frequently in postmenopausal than premenopausal women and the severity of OSA increases after menopause. Gonadal hormones can influence brain and behavior; testosterone and estrogens in particular can enhance spatial learning and memory. We hypothesized that estrogens may protect mice from IH-induced hippocampal morphological and behavioral changes. To test this hypothesis we exposed intact or gonadectomized male and female mice to room air or IH (15 cycles/h, 8 h/day, fraction of inspired oxygen (FlO₂) nadir of 5%) for a total of 30 days. During the final four days of IH mice were tested for anxiety- and depressive-like behaviors. After cessation of IH exposure mice were tested on the Barnes maze and passive avoidance tests to assess learning and memory. Ovariectomy paired with IH treatment, impaired spatial learning and memory compared to all other female groups. Intact male mice receiving IH treatment also impaired learning and memory compared to intact or castrated male mice exposed to room air. Learning and memory changes were mirrored by changes in basilar dendritic length of the CA1 region of the hippocampus. These data suggest that estrogens provide protection against IH induced deficits, whereas androgens partially exacerbate IH induced deficits on learning and memory.

Keywords: obstructive sleep apnea, intermittent hypoxia, hormones, and behavior
INTRODUCTION

Obstructive sleep apnea (OSA) is a condition characterized by repetitive upper airway obstructions resulting in cyclic intermittent hypoxia (IH) during sleep in affected individuals. OSA is an independent risk factor for diabetes, hypertension, heart disease, and stroke (25, 31, 43). In addition to health risks, people with OSA typically suffer from daytime fatigue and impaired memory (1, 26). Approximately half of people with OSA also report depression and anxiety (22).

OSA is a common health problem affecting about 9% of women and 17% of men in the US between 50-70 years or age (30). OSA is more prevalent in men than premenopausal women, but the mechanism for the sex differences remains unclear (18). Sleep apnea is also more frequently reported in postmenopausal women than premenopausal women. Even after controlling for body mass index, both the prevalence and severity of OSA increase postmenopause, and hormone replacement appears to reduce OSA severity in this population (6, 41). This observation suggests hormones as one likely mechanism underlying sex differences in OSA (18).

Sex differences are also observed in the effects of IH in mouse models of OSA. Male mice increase oxidative biomarkers following chronic IH, whereas females do not exhibit these changes (20). Additionally, oxidant production in liver mitochondria increases with ovariectomy in female rats and treatment with estradiol reduces production to levels similar to intact females (5). Patients with OSA experience drowsiness; similarly, chronic IH exposure in male mice impairs wake times and sleep latencies, whereas females maintain normal wake times and sleep latencies (37). Elevated blood pressure is common after IH exposure; gonadally-intact female rats are protected from IH-induced hypertension compared to ovariectomized females and males (15). Sex differences in ventilatory control are observed in mice following hypoxic
exposure (28) and ventilation during hypoxia is increased by exogenous progesterone treatment of newborn rat pups (4). Thus, sex differences in response to IH exist and appear to have a gonadal hormone component.

Sex hormones influence learning and memory, with both estrogens and androgens typically associated with enhanced learning and memory (12, 34). Naturally or artificially elevated estrogen concentrations in female rats enhance spatial learning in the object placement task (12). Aged, post-estropausal female mice given estradiol improve performance in the Morris water maze compared to mice without hormone replacement (11). Reproductively photoperiodic rodents display seasonal changes in hippocampal volume and spatial learning and memory such that male short day white-footed mice are impaired compared to their long day counterparts (33). Short day decreases in hippocampal volume and spatial learning and memory correspond to lower concentrations of testosterone in males and spatial learning and memory performance is enhanced in short day white-footed mice with the addition of testosterone (34). Although sex steroid hormones influence learning and memory, it remains unclear whether they influence the effects of IH on learning and memory performance.

We hypothesize that female mice are protected from IH-induced behavioral and hippocampal morphological changes in a hormone dependent manner. If this hypothesis is true, then (1) gonadally-intact females will be protected from IH-induced changes in learning and memory, as well as anxiety-like behavior compared to ovariectomized females, (2) intact females in IH will be protected compared to male mice, and (3) gonadectomized males should have similar IH-induced changes to intact females. These results could suggest hormonal manipulations that may be important in developing strategies for OSA management.

MATERIALS AND METHODS

Animals
Forty-five male and 48 female Swiss-Webster mice (~8 weeks-old) were obtained from Charles River Labs (Wilmington, MA, USA). Mice were group-housed, three to five same-sex individuals per cage in propylene cages (33 cm x 18 cm x 14 cm) at an ambient temperature of 22 ± 2 °C and relative humidity of 50% ± 10%. Mice were provided Harlan Teklad 8640 food (Madison, WI, USA) and filtered tap water ad libitum. Upon arrival mice were maintained under a 16:8 light-dark cycle of illumination for one week to allow acclimation to local conditions. Mice were then randomly assigned to either gonadectomy (n=45) or a sham-gonadectomy (n=48). Briefly, male mice were anesthetized and had a 1 cm² patch shaved on the lower abdomen. For sham-castrated male mice (n=24) testes were located and returned inside the cavity; for castration (n=21) testes were separated from the fat pad and removed, then the vas deferens and spermatic blood vessels were cauterized. All males then had their muscle and skin sutured separately. Briefly, female mice were anesthetized and had a 1 cm² patch shaved on the lower back. For sham-ovariectomized female mice (n=24) ovaries were located and returned inside the cavity; for ovariectomized mice (n=24), ovaries were separated from the fat pad and removed. All female mice then had their muscle and skin sutured separately. Following surgery, all mice were allowed to recover for two weeks before beginning air or intermittent hypoxia (IH) treatment. All experimental procedures were approved by The Ohio State University Institutional Animal Care and Use Committee.

**Hypoxia Treatment**

Mice were randomly assigned to receive IH (n=47) (15 cycles/h, 8 h/day, fraction of inspired oxygen (FI0₂) nadir of 5%) or room air (RA) (n=46) creating eight experimental groups (n=12/group, except male gonadectomized mice in RA (n=10) or IH (n=11)). FI0₂ nadir of 5% corresponds to severe OSA, classified as greater than 30 events of 10 s apneas per hour (7). Though the frequency of apneic events is reduced in the mouse model, oxygen levels are similar to those experienced by patients with severe OSA (27). Treatment occurred daily for 30
consecutive days. Mice were exposed to treatment in two 8 h shifts starting at the beginning of
the light phase; intact mice were exposed separately from gonadectomized mice. The group
that received treatment first and second shift were alternated every day. Additionally, male and
female mice were run separately; sham and ovariectomized females were exposed to treatment
on the same days and intact and gonadectomized male mice were exposed on the same days.
During treatment, mice were moved to custom-designed Plexiglas chambers (31 cm x 19 cm x
18 cm) with a raised floor (6.5 cm), 10 mice were placed in one chamber at a time (35). Oxygen
levels were controlled by connecting the cages via a regulator system to compressed air
(14L/min) and nitrogen tanks (9L/min) that automatically switched throughout exposure; levels
were checked with a flow meter (23). Before the experiment began oxygen fluctuations in the
chamber were checked using a portable data collection unit (DI-158U Series, DATAQ
Instruments, Akron, OH, USA). RA exposure mice were housed in a similar cage, without
connections to nitrogen or air tanks. Treatment occurred during the light phase (when these
animals typically sleep) and lasted for 30 days. Behavioral testing occurred during the final four
days in IH or RA treatment during the dark phase and continued after the cessation of IH
treatment.

Open Field

The open field test in mice characterizes anxiety-like responses in a novel environment, as well
as locomotor activity. Central tendency is the primary measure for anxiety-like responses, and is
defined as the proportion of time spent in the center of the open field. Locomotor activity was
measured separately as the total number of beam breaks during testing. All data were collected
from a photobeam activity system (PAS) (San Diego Instruments, San Diego, CA, USA)
contained in a chamber (Med Associates Inc., St. Albans, VT, USA). Mice were tested starting
at the beginning of the dark phase and were allowed to acclimate to the room for 20 min before
testing. Mice were tested for 20 min as previously described (9).
Barnes Maze

Mice were allowed a 24 h break between previous behavioral testing and the beginning of Barnes maze testing. The Barnes maze (San Diego Instruments, San Diego, CA, USA) assesses spatial learning and memory using a brightly light circular arena with 20 evenly spaced holes, one hole leads to a dark escape box and the other holes are blocked off with black inserts (10). Mice were acclimated to the maze on day 1, during acclimation mice were guided from the center of the maze to the target hole. Once mice were in the target hole, mice were left undisturbed for 30 sec. Then mice were trained for the next 5 consecutive days, consisting of three 90-sec trials separated by 10 min intervals in a clean cage. Latency, number of errors, and path length were averaged for the three trials each day. One day following the last training trial, mice were given a 60-sec probe trial, where they were allowed to search the Barnes maze without an escape box present. Latency to find the target hole, path length, and number of errors were scored during all trials by a video tracking program (HVS Image Ltd., Mountain View, CA, USA).

Passive Avoidance

The following day mice were tested in the passive avoidance chamber. The passive avoidance test also assesses learning and memory by allowing mice to form an association between escaping from an aversive stimulus (light) and a foot shock. Retention of this pairing was assessed 24 h after initial trial and latency to enter the dark chamber was used to assess retention. The following two days animals were placed in the passive avoidance chamber (Gemini Avoidance System, San Diego Instruments Inc., San Diego, CA). Mice were placed in the right side of the chamber in a starting box; after 20 sec the chamber was illuminated as an electrically operated door opened to expose a dark chamber on the other side. Mice had a maximum trial length of 300 sec to enter the dark chamber after which point no shock was
received and animals were removed from the apparatus. After animals entered the dark side the
door closed and mice received a 1.5 mA foot shock for 2 sec. Then mice were removed from
the chamber and returned to a clean cage. Twenty-four hours later mice were again placed in
the chamber following the same procedure. Latency to enter the dark side of the chamber was
automatically recorded by the passive avoidance chamber for both trials.

Tissue Collection and Processing

Mice were euthanized during the light phase (0700 h EST) by rapid decapitation under
isoflurane sedation. At necropsy heart, spleen, and inguinal fat pads were collected and
weighed. Brain tissue was also collected and half of each brain placed in Golgi-Cox staining
using Rapid GolgiStain Kit (FD NeuroTechnologies). The hemisphere of brain used for Golgi-
Cox staining was randomly selected. Golgi-Cox stained brains were stored, processed, and
analyzed as described previously (3). Briefly, six representative CA1 pyramidal neurons were
selected per mouse that met the following criteria: neurons were clearly stained, lacked
truncated dendrites, and were not obscured by neighboring neurons. Neurons and dendrites
were traced at 20x (0.5). Dendritic spines were traced at 100x (1.40) in 4 apical and 4 basilar,
randomly selected, representative dendritic segments of at least 20 μm in length and at least 50
μm from the cell body, using Neurolucida 8 software (MicroBrightField, Williston, VT, USA) for
PC and a Nikon Eclipse, E800 microscope. Neurons, dendrites and spine density were
analyzed using Neurolucida Explorer software (MicroBrightField, Williston, VT, USA).

Statistical Analysis

Main effect of sex (female, male) was assessed. Main effects of gonadal status (intact,
gonadectomized) and IH treatment (RA, IH) and interactions between the two factors were
assessed with data split by sex. Multivariate ANOVAs were used to analyze open field, passive
avoidance, Golgi-Cox stained dendritic morphology and organ masses, with final body mass as
a covariate. Change in body mass over the four weeks of IH or RA treatment was analyzed as a univariate ANOVA. Change in body mass across the experiment, Barnes maze latency, path length, errors, and Sholl analysis of Golgi-Cox stained tissue were analyzed with a repeated measures ANOVA. Barnes maze path length and errors were further analyzed using Mann-Whitney U nonparametric tests to assess group differences on each day due to unequal variance on individual days. Statistics were performed using SPSS 19 for Windows (IBM, New York, NY, USA). Outliers determined by Z score (±2 SEM from mean) were removed from subsequent analysis, as mentioned specifically in the results. Mean differences were considered statistically significant when $p$ was ≤0.05 and were followed up with Tukey’s honest significant differences post hoc tests, except dendritic morphology and Sholl analysis were further analyzed with least significant difference post hoc tests.

RESULTS

Open field

Male and female mice displayed similar total moves in the open field and spent a similar percentage of time in the center of the open field ($p$>0.05, in each case, Fig 1). Intact and gonadectomized female ($p$>0.05, Fig 1A) and male ($p$>0.05, Fig 1B) mice had similar levels of activity in the open field. RA and IH exposed female ($p$>0.05, Fig 1A) and male ($p$>0.05, Fig 1B) mice also had similar levels of activity in the open field. Ovariectomized female mice displayed fewer anxiety-like responses, spending a larger percent of time in the center of the open field than intact mice ($F_{1,44}=18.59$, $p$<0.01, Fig 1C). Castrated and intact male mice spent equivalent amounts of time in the center of the open field ($p$>0.05, Fig 1D). Treatment with RA or IH did not alter time spent in the center of the open field in female ($p$>0.05, Fig 1C) or male mice ($p$>0.05, Fig 1D).

Barnes Maze
Latency to find the escape hole across days of testing was similar between female and male mice ($p>0.05$, Fig 2A and B). Intact and ovariectomized mice had similar latencies to reach the escape hole across days ($p>0.05$, Fig 2A). RA and IH treated mice had similar latencies to reach the escape hole across days ($p>0.05$, Fig 2A). Gonadal status and RA/IH treatment interacted in female mice to affect latency to find the escape hole across days ($F_{1,215}=2.47$, $p<0.05$, Fig 2A), such that ovariectomized mice exposed to IH had a longer latency than intact female mice exposed to IH ($p<0.05$). Castrated mice had a shorter latency to find the escape hole than intact male mice across days ($F_{1,175}=2.75$, $p<0.05$, Fig 2B). RA exposed male mice had a shorter latency to find the escape hole than IH exposed male mice ($F_{1,175}=2.88$, $p<0.05$, Fig 2B). Gonadal status and RA/IH treatment did not interact to alter latency in male mice ($p>0.05$). Seven mice were removed from all repeated measures Barnes analysis based on a latency z score ± 2 (1 female sham RA, 2 male sham RA, 1 male sham IH, 2 male castrated RA, 1 male gonadectomized IH).

Female mice made more errors than males across days of training in the Barnes maze ($F_{5,390}=4.59$, $p<0.01$, Fig 2C and D respectively). There were no main effects on the number of errors made or path length in female or male mice across days; however, due to sex differences and differences in latency we examined differences on each individual day of testing. Intact female mice made fewer errors than ovariectomized mice on days 2 ($U=161$, $p<0.05$), 5 ($U=140$, $p<0.05$) and 6 ($U=124$, $p<0.05$, Fig 2C). RA or IH treatment did not alter the number of errors made by female mice on days 1-6 ($p>0.05$, Fig 2C). Castrated and intact male mice did not alter the number of errors made on days 1-6 ($p>0.05$, Fig 2D). RA exposed male mice made fewer errors than IH exposed male mice on days 1-6 (day 1 $U=114$; day 2 $U=65$; day 3 $U=54$; day 4 $U=19$; day 5 $U=46$; day 6 $U=63$, $p<0.05$ in each case, Fig 2D). Path length to reach the escape hole across days of testing was similar between female and male mice ($p>0.05$, Fig 2E and F respectively). Intact female mice had a shorter path length than ovariectomized mice on
days 2 \((U=107, p<0.05)\), 5 \((U=150, p<0.05)\) and 6 \((U=137, p<0.05, \text{Fig } 2E)\). IH exposed female
mice had a shorter path length than RA exposed female mice on days 4 \((U=176, p<0.05)\) and
5 \((U=171, p<0.05, \text{Fig } 2E)\). Castrated and intact male mice had similar path lengths on days 1-6
\((p>0.05, \text{Fig } 2F)\). IH exposed male mice had a longer path length than RA exposed male mice
on days 1-6 (day 1 \(U=98\); day 2 \(U=62\); day 3 \(U=62\); day 4 \(U=21\); day 5 \(U=54\); day 6 \(U=67,\)
\(p<0.05\) in each case, \text{Fig } 2F).

Male (\text{Fig } 3B) mice made less pokes in the escape hole during the probe trial of the Barnes
maze than female mice \((F_{1,85}=6.5, p<0.01, \text{Fig } 3A)\). Gonadectomized and intact female \((p>0.05,\)
\text{Fig } 3A) and male \((p>0.05, \text{Fig } 3B)\) mice made a similar number of pokes in the escape hole
during the probe trial. Female \((p>0.05, \text{Fig } 3A)\) and male \((p>0.05, \text{Fig } 3B)\) mice exposed to RA
and IH made a similar number of pokes in the escape hole during the probe trial. Male and
female mice spent a similar percentage of time in the correct quadrant during the probe trial of
the Barnes maze \((p>0.05, \text{Fig } 3C\text{ (females) and } D\text{ (males)}\). Ovariectomized mice spent a
smaller percentage of time in the correct quadrant of the Barnes maze than intact female mice
\((F_{1,44}=11.01, p<0.01, \text{Fig } 3C)\). Female mice exposed to RA and IH spent a similar percent of
time in the correct quadrant of the Barnes maze \((p>0.05, \text{Fig } 3C)\). Gonadal status and treatment
interacted to alter time spent in the correct quadrant of the Barnes maze in female mice such
that intact mice in RA and IH spent similar amounts of time in the correct quadrant of the Barnes
maze, whereas ovariectomized mice in IH spent less time in the correct quadrant than
ovariectomized mice in RA \((F_{1,44}=22.87, p<0.01, \text{Fig } 3C)\). Castrated mice spent a smaller
percentage of time in the correct quadrant of the Barnes maze than intact male mice \((F_{1,41}=4.31,\)
\(p<0.05, \text{Fig } 3D)\). Male mice in IH spent less time in the correct quadrant of the Barnes maze
than mice exposed to RA \((F_{1,41}=5.56, p<0.05, \text{Fig } 3D)\). Gonadal status and treatment did not
interact to alter percent of time in the correct quadrant during the probe trial in male mice
\((p>0.05)\).
Passive Avoidance

All groups had similar latencies to enter the dark chamber on day 1 ($p>0.05$ in each case, Fig 4 A (females) and B (males)). Males had a reduced latency to enter the dark chamber on the second day of passive avoidance compared to female mice, indicating reduced learning and memory ($F_{1,85}=26.09$, $p<0.01$, Fig 4 C (females) and D (males)). Intact and gonadectomized female ($p>0.05$, Fig 4C) and male ($p>0.05$, Fig 4D) mice had similar latencies to enter the dark chamber on the second day of passive avoidance. Similarly, female ($p>0.05$, Fig 4C) and male ($p>0.05$, Fig 4D) mice exposed to RA and IH had similar latencies to enter the dark chamber on the second day.

Body Mass

Changes in body mass during the four weeks of RA or IH treatment were similar in male and female mice ($p>0.05$ in each case, Table 1). Ovariectomized mice had a similar change in body mass as intact female mice ($p>0.05$, Table 1). Castrated mice had a similar change in body mass as intact female mice ($p>0.05$, Table 1). Mice in RA gained more mass than mice in IH for both female ($F_{1,44}=49.09$, $p<0.01$, Table 1) and male mice ($F_{1,40}=105.76$, $p<0.01$, Table 1).

Gonadal status and treatment interacted in female mice to alter change in body mass ($F_{1,44}=80.53$, $p<0.01$, Table 1), such that intact female mice in IH had a greater decrease in body mass than ovariectomized mice in IH.

Female mice had a lower body mass than male mice across the experiment ($F_{5,415}=2.82$, $p<0.05$, Fig 5A and B respectively). Ovariectomized mice had a larger mass than intact female mice across the experiment ($F_{5,220}=9.61$, $p<0.01$, Fig 5A). Female mice in IH had lower body mass than female mice in RA treatment across weeks of the experiment ($F_{5,220}=145.3$, $p<0.01$, Fig 5A). Gonadal status and RA/IH treatment interacted to alter body mass across the experiment ($F_{5,220}=14.7$, $p<0.01$, Fig 5A), such that intact female mice in IH lost more mass than
ovariectomized mice. Castrated mice had a smaller body mass than intact male mice across the experiment ($F_{5,200}=15.9, p<0.01$, Fig 5B). Male mice in IH had lower body mass than male mice in RA treatment across weeks of the experiment ($F_{5,200}=42.8, p<0.01$, Fig 5B). Gonadal status and RA/IH treatment did not interact to alter body mass across the experiment in male mice ($p>0.05$, Fig 5B). One mouse was removed because it was an outliers based on Z score (one male sham mouse in IH).

Organ Masses

Male mice had larger heart mass than female mice ($F_{1,84}=27.73, p<0.01$, Table 2). Male and female mice had similar spleen masses ($p>0.05$). Female mice had larger fat pad mass than male mice ($F_{1,84}=19.55, p<0.01$, Table 2). Ovariectomized mice had similar heart, spleen, and fat pad masses compared to intact female mice ($p>0.05$, Table 2). RA and IH exposed female mice had similar heart, spleen, and fat pad masses compared to intact female mice ($p>0.05$, Table 2). Castrated mice had similar heart, spleen, and fat pad masses compared to intact male mice ($p>0.05$, Table 2). RA and IH exposed male mice had similar heart, spleen, adrenal gland, and fat pad masses compared to intact male mice ($p>0.05$, Table 2).

Dendritic Morphology

Male and female mice had similar apical and basilar lengths and spine densities ($p>0.05$ in each case, Fig 6A-H). Male mice had a larger cell body area than female mice ($F_{1,84}=4.6, p<0.05$, Fig 6J and I respectively). Neither gonadal status nor RA/IH treatment altered apical spine density in female mice ($p>0.05$ in each case, Fig 6A). Ovariectomized mice increased basilar spine density compared to intact female mice ($F_{1,38}=5.51, p<0.05$, Fig 6C); basilar spine density was not altered by RA or IH treatment ($p>0.05$). Gonadal status and RA/IH treatment did not alter apical dendritic length in female mice ($p>0.05$ in each case, Fig 6E). Gonadal status and RA/IH treatment alone did not alter basilar dendritic length in female mice ($p>0.05$ in each case, Fig
Gonadal status and treatment interacted to alter basilar dendritic length in female mice ($F_{1,38}=9.01$, $p<0.01$, Fig 6G), such that ovariectomized mice exposed to IH reduced basilar dendritic length compared to ovariectomized mice exposed to RA and intact female mice exposed to RA ($p<0.05$). Cell body area was not altered by gonadal status or RA/IH treatment in female mice ($p>0.05$ in each case, Fig 6I). Gonadal status and RA/IH treatment did not alter apical spine density in male mice ($p>0.05$ in each case, Fig 6B). Gonadal status and RA/IH treatment did not alter basilar spine density ($p>0.05$ in each case, Fig 6D). Gonadal status and RA/IH treatment did not alter apical dendritic length in male mice ($p>0.05$ in each case, Fig 6F). Gonadal status and RA/IH treatment did not alter basilar dendritic length in male mice ($p>0.05$ in each case, Fig 6H). Cell body area was not altered by gonadal status or RA/IH treatment alone in male mice ($p>0.05$ in each case, Fig 6J). Gonadal status and RA/IH treatment interacted to alter cell body area in male mice ($F_{1,38}=5.55$, $p<0.05$, Fig 6J), such that intact male mice in RA decreased cell body area compared to castrated mice in RA ($p<0.05$). Nine mice were not included in any analysis of neuronal morphology because there was insufficient staining of the tissue or the brain was damaged during processing (1 female sham RA, 1 female sham IH, 2 female ovariectomized RA, 2 female ovariectomized IH, 1 male sham RA, and 2 male gonadectomized IH).

Female mice had a similar number of branches at each apical and basilar radii as male mice ($p>0.05$, Fig 7A and C and E and G respectively). Ovariectomized and intact female mice had similar apical radii ($p>0.05$, Fig 7A). IH treatment decreased apical radii compared to RA treatment in female mice ($F_{45,1710}=2.04$, $p<0.05$, Fig 7A and B). Castrated mice increased apical radii compared to intact male mice ($p>0.05$, Fig 7C and D). IH and RA treatment did not alter apical radii in male mice ($p>0.05$, Fig 7C). Ovariectomized and intact female mice had similar basilar radii ($p>0.05$, Fig 7E). IH treatment decreased basilar radii compared to RA treatment in female mice ($F_{33,1254}=1.45$, $p=0.05$, Fig 7E and F). Gonadal status and RA/IH treatment
interacted to alter basilar radii in female mice ($F_{33.1254}=3.04$, $p<0.05$, Fig 7E), such that
ovariectomized mice exposed to IH decreased basilar radii compared to ovariectomized RA
exposed mice ($p<0.05$). Neither gonadal status nor treatment altered basilar radii in male mice
($p>0.05$ in each case, Fig 7G).

**DISCUSSION**

OSA is more prevalent and severity seems to be affected in a hormone dependent
manner such that more men are affected than premenopausal women, although the sex
difference in both prevalence and severity wanes when postmenopausal women are compared
to men (6, 18). Intact female mice are protected from the negative cellular consequences of IH
and ovariectomy removes this protection (15). Additionally, gonadal hormones can enhance
spatial learning and memory in mice. Therefore, we hypothesized that estrogens protect mice
from IH induced behavioral and hippocampal morphological changes. IH reduced body mass as
previously described, indicating model validity (2). Ovariectomy paired with IH treatment,
impaired spatial learning and memory compared to all other female groups. Intact male mice
receiving IH treatment also significantly impaired learning and memory compared to intact or
castrated male mice exposed to RA. Basilar dendritic length in female mice was reduced in
ovariectomized and IH exposed mice compared to intact females exposed to IH, similar to
changes observed in Barnes maze learning and memory. Additionally, IH treatment in female
mice reduced branching in both apical and basilar dendrites compared to RA. These data
suggest that estrogens provide protection against IH induced deficits, whereas androgens
partially exacerbate IH induced deficits.

Just as patients with OSA have impaired cognition, IH induces impairments in the
Barnes maze (8, 36, 42). Both estrogens and androgens, are typically associated with
enhanced learning and memory (12, 34). However, despite the increase in prevalence of OSA
in men compared to women and the increase in prevalence of OSA in postmenopausal women, the influence of estrogens and androgens is not widely studied. As mentioned, intact female mice are protected from the negative cellular consequences of IH and ovariectomy removes this protection (15). Similarly, intact female mice were protected against the IH-induced spatial learning and memory deficits observed in ovariectomized mice. Intact male mice in IH had impaired learning and memory compared to all RA exposed male mice. Estrogens and androgens are associated with enhanced learning and memory (12, 34). Although hormone concentrations were not assessed in the current study there are previously-reported hormone-dependent sex differences in response to IH. Because overall IH exposed mice exhibited similar latencies and path lengths as RA exposed mice, activity differences are probably not present after the cessation of daily IH exposure. Observed differences in learning and memory are likely independent of the effects of sleep deprivation. Sleep architecture during the initial days of IH treatment is disrupted, but normalized by the end of the 14 day treatment (13); however, rats exposed to IH still exhibit impairments in the Morris water maze suggesting a role for IH in impairing learning and memory independent of sleep deprivation (13). These data suggest a protective role for estrogens against IH induced spatial learning and memory deficits, whereas androgens may exacerbate IH-induced deficits in spatial learning and memory.

The passive avoidance test did not indicate learning and memory differences. However, passive avoidance was tested a week after removal from IH or RA treatment, possibly the negative consequences are reversible and prolonged removal from IH allows recovery of function. Additionally, passive avoidance requires amygdal-based fear learning, whereas spatial learning and memory is hippocampus based. The Barnes maze requires learning and retention over a seven day span, whereas the passive avoidance task examined retention 24 h later. These results suggest that IH and gonadal status may not affect shorter-term memory, but might influence long-term memory consolidation (40, 45).
Given that changes in learning and memory are associated with the hippocampus, we examined changes in hippocampal dendritic morphology. IH-induced changes in hippocampal morphology can return to normoxic levels following 21 days of IH exposure (24, 39). Changes can occur with prolonged exposure to IH. Reduced apical spine density in the CA3 was observed in a similar IH set up with 21 days of 8h/day IH exposure (2). In the current study IH decreased apical and basilar dendritic branching in female mice in *cornu Ammonis* (CA) 1. Similarly, basilar dendritic length was reduced in ovariectomized mice exposed to IH compared to ovariectomized RA exposed mice and intact female mice exposed to IH. These changes in basilar dendritic length correspond to the decreased spatial learning and memory observed in the ovariectomized mice exposed to IH. IH based changes in dendritic morphology corresponding to learning and memory deficits were not observed in male mice. One consideration for these results is that collection of the brains occurred two weeks after removal from IH or RA treatment. The timing of tissue collection gave mice ample time in normoxic conditions for dendritic morphology to completely or partially recover, as dendritic morphology changes rapidly differences might have been missed (17, 29). Dendritic morphology was affected in a hormone specific manner in female mice.

Hormonal influences from estrous cycles, gonadectomy, IH, and food composition must be considered in the current experiment. A recent meta-analysis suggests that variability on individual measures was the same for randomly cycling females as males; the results suggest constant monitoring of estrous cycles is unnecessary when using female mice (32). Female performance on the T-maze is not altered by proestrus or estrus; whereas castration decreases accuracy during training (14). Similarly, ovariectomy is associated with memory impairments compared to intact females (38). A few days of IH exposure increases testosterone (16); however, a 17 day exposure to IH reduces testosterone concentrations compared to male mice in normal air conditions (44). Many changes associated with short IH exposure alone normalize
over prolonged exposure, such as changes in corticosterone (2) or dendritic morphology (24); a similar phenomenon may occur with testosterone concentrations. The Harlan 8640 diet used in this study has soybean meal, containing phytoestrogens that can act as both an estrogen agonist and antagonist. A diet rich in phytoestrogens improves visual spatial memory performance in females compared to a diet free of phytoestrogens, whereas, males perform better on the diet free of phytoestrogens (19). Similarly, ovariectomized females on a diet rich in phytoestrogens improve performance on a spatial memory task and increase spine density in the CA1 region of the hippocampus (21). Because all of the mice received the same diet, diet is not likely a factor in group differences within sexes and no differences in Barnes maze were observed between the sexes. However, diet may explain the reduced learning and memory performance observed in males compared to females on the passive avoidance test. Overall, comparing both sexes likely provides the most accurate picture of hormone dependent effects of IH exposure.

In conclusion, these data suggest cycling female sex hormones provide protection against IH induced deficits, whereas androgens partially exacerbate IH induced deficits in male mice. Our conclusions support clinical observations that women seem to be protected from OSA until menopause and that hormone replacement following menopause reduces severity of OSA (6, 41). Additionally, these data suggest that hormone replacement might be an important intervention to help ameliorate cognitive impairments associated with OSA particularly in postmenopausal women.

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. Total activity in the open field was not altered by gonadal status or treatment in female (A) or male (B) mice. Percent of time spent in the center of the open field was higher in ovariectomized mice than intact female mice (C), indicating increased anxiety-like behavior. Mean (±SEM) total moves in the open field for female (A) and male mice (B). Mean (±SEM) percent of time in the center of the open field, ovariectomized female mice spent a larger percent of their time in the center of the open field than intact mice (C). Mean (±SEM) percent of time in the center of the open field did not differ among male mice (D). Significant mean differences at $p<0.05$ indicated by different letters above groups (e.g., a vs b) as determined by Tukey HSD post hoc. RA= room air, IH= intermittent hypoxia, OVX= ovariectomized, CAST = castrated.

Figure 2. Intact female mice were protected against IH-induced spatial learning and memory deficits, whereas, intact male mice exposed to IH had impaired learning and memory compared to RA exposed males. Mean (±SEM) latency to find the escape hole for female mice (A), number of errors for female mice (C), and path length for female mice (E). Mean (±SEM) latency to find the escape hole for male mice (B), number of errors for male mice (D), and path length for male mice (F). Significant mean differences among groups at $p<0.05$ indicated by asterisk (*) as determined by Tukey HSD post hoc. RA= room air, IH= intermittent hypoxia, Gonadx = gonadectomized.

Figure 3. Ovariectomized mice reduced time spent in the correct quadrant of the Barnes maze during the probe trial compared to intact female mice, particularly in IH exposed mice (C). Male mice exposed to IH reduced time spent in the correct quadrant of the
Barnes maze during the probe trial compared to RA exposed mice (D), indicating impaired memory. Mean (±SEM) number of nose pokes into the escape hole in female (A), and male mice (B). Mean (±SEM) time spent in the correct quadrant of the Barnes maze during the probe trial in female mice (C) and male mice (D). Significant mean differences at $p<0.05$ indicated by different letters above groups (e.g., a vs b) as determined by Tukey HSD post hoc. RA= room air, IH= intermittent hypoxia, OVX = ovariectomized, CAST = castrated.

Figure 4. Male mice had impaired learning and memory in the passive avoidance test compared to female mice. Latency to enter the dark side of the chamber on the first day of testing in female (A) and male mice (B). Latency to enter the dark side of the chamber on the second day of testing in female (C) and male mice (D). Significant mean differences at $p<0.05$ indicated by different letters above groups (e.g., a vs b) as determined by Tukey HSD post hoc. RA= room air, IH= intermittent hypoxia, OVX = ovariectomized, CAST = castrated.

Figure 5. Male mice had a larger body mass than female mice. Male and Female mice in IH had a lower body mass than mice exposed to RA. Ovariectomized females increased body mass compared to intact mice (A), whereas, castrated mice decreased body mass compared to intact mice (B). Mean (±SEM) female (A) and male (B) body mass across the experiment. Significant mean differences at $p<0.05$ from all other groups indicated by asterisk (*), mean differences from mice exposed to IH indicated by caret (^), other mean differences among groups indicated by pound sign (#). RA= room air, IH= intermittent hypoxia, Gonadx = gonadectomized.

Figure 6. Overall, male mice had an increased cell body area compared to female mice as assessed by Golgi-Cox staining (J and I respectively). Ovariectomy increased basilar spine density compared to intact female mice (C). Gonadal status and RA/IH treatment interacted in female mice to affect basilar dendritic length (G) such that ovariectomized female mice in IH
decreased basilar dendritic length compared to intact mice exposed to IH. Gonadal status and RA/IH treatment interacted in male mice to affect cell body area (J) such that intact male mice exposed to IH increased cell body area compared to RA but this did not occur in castrated mice.

Mean (±SEM) apical spine density in female (A) and male mice (B), basilar spine density in female (C) and male mice (D), length of apical dendrites in female (E) and male mice (F), length of basilar dendrites in female (G) and male mice (H), and cell body area in female (I) and male mice (J). Representative neuron in the CA1 region, from a CAST male in IH, apical and basilar dendrites, cell body and spine density indicated (K). This image was captured at 20x, spine density inlay taken at 100x (K). Significant mean differences at $p<0.05$ indicated by different letters above groups (e.g., a vs b) as determined by LSD post hoc test. RA= room air, IH= intermittent hypoxia, OVX = ovariectomized, CAST = castrated.

**Figure 7.** Overall IH treatment in female mice reduced the number of apical (B) and basilar (F) branches indicated by Sholl analysis. Castration overall increased apical branching (D). Gonadal status and RA/IH treatment interacted in female mice (E) such that ovariectomized mice exposed to RA increased the number of basilar branches compared to ovariectomized mice exposed to IH (determined by LSD post hoc test). The number of apical branches at each radius in female mice (A), comparing grouped RA and IH in female mice (B). The number of apical branches at each radius in male mice (D), comparing grouped intact and castrated mice (D). The number of basilar branches at each radius in female mice (E), comparing grouped RA and IH in female mice (F). The number of basilar branches at each radius in male mice (G). Significant mean differences at $p<0.05$ indicated by asterisk (*). RA= room air, IH= intermittent hypoxia, Gonadx = gonadectomized.

References


**Table 1.** Mean (± SEM) change in body mass (g) between the start and end of the four weeks of room air (RA) or intermittent hypoxia (IH) treatment. RA = room air; IH = intermittent hypoxia; OVX = ovariectomized; CAST = castrated.

<table>
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<tr>
<th>Gonadal Status</th>
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<tr>
<td><strong>Females</strong></td>
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<tr>
<td>Intact (sham)</td>
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<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact (sham)</td>
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</tr>
<tr>
<td>CAST</td>
<td>0.98 ± 0.30</td>
<td>-1.16 ± 0.28</td>
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Table 2. Mean (± SEM) organ masses with final body mass (g) as a covariate. Data presented are from after four weeks of room air (RA) or intermittent hypoxia (IH) treatment and 13 days after removal from RA and IH treatments. RA = room air; IH = intermittent hypoxia; OVX = ovariectomized; CAST = castrated.

<table>
<thead>
<tr>
<th>Tissue</th>
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<th>Males (g)</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
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<tr>
<td></td>
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<td>0.166±0.025</td>
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<tr>
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<tr>
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<td>0.867±0.104</td>
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