ANDROGEN MODULATION OF CNS DURING CHRONIC INTERMITTENT HYPOXIA

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DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, Texas
April 2018

ACKNOWLEDGEMENTS

I would like to thank Dr. Rebecca L. Cunningham for her excellent mentorship over the past five years. She has encouraged me to expand my skills and knowledge in the lab beyond what I had originally imagined. She has also provided a multitude of opportunities to develop competence in public and written communication. Her insight is always valued and her sense of humor greatly appreciated.

I would also like to thank the members of my committee, Dr. Robert Barber, Dr. J. Thomas Cunningham, Dr. Derek Schreihofer, and my University Member, Dr. John Planz. They have proved to be excellent mentors in their own right, and their guidance opened up new avenues of investigation along the way. Their expertise is always welcome.

I would also like to thank my parents and sisters for encouraging me to continue to pursue doctoral studies. They are always close, no matter how far apart we live. The women in my life who stopped occasionally to share a run or a cup of coffee and a word of encouragement have made this journey seem much easier. And last, but never least, I would like to thank my sons, Sterling and Conner, for being patient with a mom trying to juggle her own school assignments with theirs, and my husband, John, who has made this endeavor possible.

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SPECIFIC AIMS

Alzheimer's and Parkinson's diseases are the two most commonly diagnosed neurodegenerative disorders in the United States. Alzheimer's disease (AD) was the 6th leading cause of death and Parkinson's disease was the 14th in 2014. By the time patients present with clinical symptoms of these neurodegenerative diseases (ND), there is no cure to halt the progression. Age is the greatest risk factor for these diseases. As the baby boomer generation ages, the incidence of these diseases is expected to increase dramatically, providing an economic and caregiver burden to our economy. Sex differences exist in both the onset and progression of ND, indicating sex hormones may play a role in ND.

Hallmarks of both AD and PD are preclinical elevations in oxidative stress (OS) and inflammation in circulation, cerebrospinal fluid, and brain regions impacted by each disease. A variety of genetic and environmental factors have been documented to contribute to elevated OS and inflammation. Basal OS increases with age and can activate inflammatory processes. Inflammation, in turn, can elevate OS and trigger transcription of pro-apoptotic genes. It has been proposed that there is an OS threshold that impairs the body's homeostatic processes, leading to increased ND risk.

One contributor to OS is obstructive sleep apnea (SA). SA is a common comorbidity of ND. Repetitive hypoxic events during sleep is a key component of SA. Our model of chronic intermittent hypoxia (CIH) has been associated with elevated OS and inflammation systemically, as well as in brain regions known to be impacted during early stages of ND. The incidence of SA increases with aging and occurs more frequently in men than women, suggesting the major male hormone, testosterone (T) may contribute to the effects of SA.

Testosterone replacement therapy (TRT) has become a popular therapeutic over the past decade. However, most studies on the beneficial aspects of TRT have been performed in young, hypogonadal men, and the long-term effect of TRT on aging has not been well investigated. Previous studies in our lab indicates that testosterone (T) plays a dual role in OS. When T is applied to neurons prior to OS exposure, it is protective against subsequent OS insults. However, under OS conditions, T exacerbates the apoptotic effect of OS, increasing cell death. Interestingly, in male rats exposed to the oxidative stressor, CIH, plasma OS is elevated. This suggests a mechanism by which T can exacerbate OS and the subsequent the risk of ND.

LONG TERM GOALS: Identify early contributors to neurodegeneration to improve preventative treatment options.

OBJECTIVE: Determine the role androgens play in the risk of developing neurodegeneration in males.

HYPOTHESIS: **Under chronic intermittent hypoxia, testosterone leads to an elevation of OS and inflammation in brain regions affected during early-stage neurodegeneration.**

AIM #1: Determine the conditions under which testosterone is protective against neurodegeneration.

AIM #2: Determine the conditions under which testosterone is damaging to neurons.

CHAPTER I

SEX DIFFERENCES IN SLEEP APNEA AND COMORBID NEURODEGENERATIVE DISEASES

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**published in* STEROIDS. 2017 Dec 16. pii: S0039-128X(17)30232-5

Abstract

Sleep apnea is a disorder, which increasingly affects people worldwide. Whether the associated hypoxic events during sleep are central or obstructive in origin, the end result is excessive daytime sleepiness and an increased risk for several comorbidities, such as cardiovascular and neurodegenerative disorders. Sleep apnea is diagnosed more frequently in men than women, suggesting a role of sex hormones in the pathology of the disease. Furthermore, there are sex differences in the development and progression of comorbid diseases associated with sleep apnea. Therefore, treatment of sleep apnea may be clinically relevant for prevention of subsequent sex-specific comorbid disorders. While the impact sleep apnea has on cardiovascular events has been the subject of many research studies, the role of sleep apnea in neurodegeneration is less established. Here we review known risk factors for sleep apnea and the implications of the observed sex differences in this disease. We also summarize the evidence and mechanisms for how sleep apnea may contribute to the onset of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease.

Sleep apnea is estimated to affect 26% of the population in the United States (78), although up to 80% of those with the disorder are undiagnosed (50). The major outcome of this condition is repetitive reduction in inspired oxygen during sleep (27). This may be due to either a loss of central control of breathing effort or a physical obstruction of the upper airway (27), leading to apneas and/or hypopneas. One measure of the severity of sleep apnea is the apnea/hypopnea index (AHI), which quantifies the number of times per hour an apnea or hypopnea occurs. Cutoff points for the diagnosis of mild, moderate, and severe sleep apnea are AHI ≥ 5, ≥ 15, and ≥ 30 respectively (86). Treatment is considered necessary for cases of moderate and severe sleep apnea, but optional for mild sleep apnea (32). The most common therapeutic options include use of an oral apparatus or continuous positive airway pressure (CPAP) to maintain an open airway during sleep (32, 57).

Sleep apnea comorbid disorders include neurodegenerative disorders, such as Alzheimer's disease (AD) or Parkinson's disease (PD). These types of diseases are progressive disorders, which lead to a loss of neurons in specific areas of the central nervous system. Significant loss of neurons results in a functional deficit associated with the affected region. The initial degradation is a slow insidious process, wherein the patient is often unaware of the disorder. Clinical symptoms of AD include loss of working and episodic memory, followed by impaired executive functions and eventual autonomic system deficits (67). These do not become apparent until approximately 40% damage to neurons in hippocampal structures occurs (13). PD symptoms of bradykinesia, tremor, rigidity, and postural instability (59) do not manifest until 70-80% of neurons in the substantia nigra are lost (15). Because diagnosis generally occurs during advanced stages of the disease, therapeutic treatments are generally not efficacious (11, 14, 21,

56, 97). Identification of modifiable risk factors which exacerbate neurodegeneration may prove to be crucial to improving therapeutic options. One of these factors may be sleep apnea.

The association between sleep apnea and neurodegeneration has only recently begun to be explored, but current evidence suggests people with sleep apnea are at higher risk for neurodegenerative diseases (27). For example, Peng, et al., recorded deficient regional brain activity in men with severe obstructive sleep apnea, connecting this sleep disorder with pathologic neural consequences (77). Sleep apnea has been associated with deficits in working memory (33) and overnight consolidation of motor skill acquisition (58). Evidence exists to support the hypothesis sleep apnea can increase the risk of developing AD or PD (23, 33, 73, 76, 102). In a multi-ethnic study, people who carried a genetic predisposition for AD exhibited a significant decline in cognition as hypoxic events increased in severity (47). Additionally, sleep apnea is linked with increased risk for sporadic PD (102). Therefore, treatment of sleep apnea may improve cognitive deficits in people with AD and PD (1, 25, 41, 74).

An increase in sleep disorders has been reported in association with both AD and PD (3, 17, 22, 23, 28, 29, 47, 48, 54, 63, 95, 102). In our studies using the Texas Alzheimer's Research Care and Consortium cohort (TARCC, a collaborative Alzheimer's research effort funded by the State of Texas), we found participants with cognitive deficits are more likely to report a significant elevation in sleep disturbances than those who are cognitively intact or have mild cognitive impairment (figure 1, table 1). As dementia severity increases, so do sleep disturbances, which is evidenced by the higher number of sleep disturbances in AD patients versus mild cognitive impairment patients. Similar to our observations, other studies have documented an increased incidence of sleep apnea as the severity of PD progresses (20, 23, 90). Although a few studies have not found the same association between sleep apnea and

neurodegeneration (3, 29, 63), small sample sizes could be a contributing factor to this discrepancy. In general, literature indicates that sleep apnea increases the risk of neurodegeneration. However, it is unknown whether sleep apnea is a causative agent of AD or PD.

Sex Differences in Sleep Apnea

Men are 2 – 3 times more likely to be diagnosed with sleep apnea than women, and the incidence in both sexes increases dramatically with age (78, 80, 101, 103). This suggests sex hormones play a role in the development of sleep apnea. Estimates of the prevalence of sleep apnea indicate sex differences occur worldwide. In the United States, 24% of men and 9% of women are reported to experience an AHI $\geq$ 5 (103). Similar results are observed in investigations conducted in Europe, Asia, and South America (78, 80, 87, 92, 93). Several basic science studies indicate that biological sex differences are involved in the manifestation and progression of sleep apnea.

Sex has an impact on other risk factors, such as age and body weight, which can affect the onset and severity of sleep apnea. Men with sleep apnea are at higher risk for comorbid events than women (101), and have higher risk for sleep apnea during middle age than women (8). In addition to more frequent incidence with age, men, unlike women, experience increasing AHI severity as they age (68, 78, 80). Women diagnosed with sleep apnea are not only older at initial diagnosis, they are also diagnosed with less severe AHI than their male counterparts (8). While the prevalence appears to increase as women enter menopause, the severity of AHI events does not (78, 103). Interestingly, young women with polycystic ovary syndrome (PCOS), characterized by high testosterone levels, are at higher risk to develop obstructive sleep apnea

(44). This suggests that sex hormones (e.g. androgens and estrogens) may underlie these sex differences in sleep apnea onset, progression, and severity.

Sex-specific anatomical differences in adipose tissue deposition and airway size may account for some of the discrepancies in sleep apnea prevalence and severity. It has been postulated fat deposition around the neck contributes to airway constriction (27, 31, 71). Sex hormones are crucial in determining the composition and deposition of adipose tissue, as well as the size of airway structures (89). The result of this is a higher deposition of fat in upper body areas in men, such as around the neck and in the thoracic abdominal region, as opposed to women who are more likely to carry weight in their lower abdomen (101). Interestingly, obesity and anatomical features contribute to the development and severity of sleep apnea in men, but not women (78, 80). Men experience a positive correlation between AHI severity and all obesity measures, such as body mass index (BMI), waist-hip ratio, or neck circumference, regardless of age. The severity of AHI can be reduced by weight loss (8). Neck circumference, narrow airway structure, and loss of muscle tone in a supine position can all be indicators of risk in men (8, 71).

Unlike men, associations between obesity and sleep apnea are not observed in women. Pre-menopausal women with sleep apnea tend to be more obese at lower AHI's than men, and there is not an association between BMI and AHI severity (8). Additionally, sleep apnea severity is not dependent on waist-hip ratios or neck circumference in women or affected by weight loss (80). Although the prevalence of sleep apnea increases in post-menopausal women compared to pre-menopausal women, an association between obesity and sleep apnea is still not present in post-menopausal women, unlike what is observed in men (8, 101). Pre-menopausal women do not experience the functional loss of airway musculature in a supine position observed in men (71), despite smaller anatomical structure. It is only post-menopausal women who experience a

loss in respiratory function, which may be one contributor to the age-related increase in prevalence observed in women. Women with PCOS exhibit altered fat composition and deposition, which closely resembles patterns observed in men (82). However, no studies have examined the impact of airway function on sleep apnea in PCOS women. The change in adipose tissue to resemble male characteristics may partially account for the elevated risk of sleep apnea in these women, and is further support for the idea sex hormones are contributing to observed sex differences.

In addition to distinctive anatomical differences, men and women with sleep apnea report different symptoms to primary care physicians (8, 50, 60, 81, 101). Among patients referred for diagnostic sleep studies, men are more likely to report snoring and witnessed accounts of apneas and nocturnal gasping (8). Women diagnosed with sleep apnea initially present with complaints of daytime sleepiness, fatigue or lack of energy, morning headaches, memory impairments, and enuresis more frequently than men do (8, 60, 81). During sleep study assessments, men exhibit high AHI events during non-REM sleep phases (71). Conversely, women are more likely to experience AHI events during both REM and non-REM sleep phases in conjunction with a lower supine AHI overall (71). This results in more mild diagnoses of sleep apnea for women.

It is intriguing that each of the above risk factors can be modulated by sex hormones. Due to the patterns observed in middle aged men and women, sex hormones may differentially confer protection or exacerbate risk of sleep apnea and associated comorbidities (2, 34). Sex hormones peak during young adulthood and then decline at different rates in men and women as they age. Testosterone and estrogen levels gradually decline as men age (2, 9, 79). In contrast, estrogen drops abruptly in women as they enter menopause, while testosterone levels remain stable (24). In men, the decline in testosterone levels has been associated with adiposity, less efficient sleep,

and increased risk for cardiovascular and neurodegenerative events (2, 6, 7). The role testosterone plays in the mechanisms of sleep apnea is the subject of current discussion and research studies within the aging and endocrinology fields (6, 39, 40). Further investigation into the role of androgens and estrogens in the mechanisms of this disease are vital to determine how hormone therapy may differentially impact sleep apnea and its associated comorbidities in men and women.

Sex Differences in Neurodegeneration

Sex differences exist in the risk and symptoms of neurodegeneration, which parallel the pattern observed in sleep apnea. Aging, which is implicated in elevated incidence and severity of sleep apnea, is the primary risk factor for developing both AD and PD (59, 65). While the risk for men to develop AD begins during middle age and increases linearly with aging, women appear to be protected from AD until reaching menopause (100). At this point, the incidence rate in women climbs steeply until it reaches the same level as men. Interestingly, young women with PCOS are at higher risk to develop AD than young women without PCOS (18, 83), and appear to present a similar pattern of developing AD as men, supporting a role for androgens. Following transition from MCI to AD, men appear to have slower cognitive decline than women with AD, suggesting sex hormones impact disease progression (70). Similarly, men are 1.3 to 2 times more likely to develop PD than women, are diagnosed at a younger age, and are more likely to experience motor deficits in their face, neck, and arms (4, 59, 96). Women with PD suffer more often from tremor, postural deficits, and depression (59). Due to the similarities between men and women with PCOS, it is likely hormones, as opposed to genetics, are major contributors to the patterns observed in sleep apnea and the onset of neurodegeneration.

Common Mechanisms and Interactions Between Sleep Apnea and Neurodegenration

Using the Texas Alzheimer's Research Care and Consortium (TARCC) cohort, we found men more frequently report sleep disturbances than their female counterparts (figure 1). This is similar to observations in other studies, in which men with PD report more daytime sleepiness than women with PD (59). Post-mortem examination of androgens and estrogens in the brains of AD patients show men have a steeper decline in androgens, but not estrogens, than their healthy counterparts (84). Men with PD also frequently experience a decline in bioavailable testosterone (69). Women do not exhibit similar effects in either hormone. These observations support the hypothesis androgens may be fundamental to the mechanisms by which sleep apnea heightens the risk of neurodegeneration.

Hypertension, elevated inflammation, and oxidative stress are all common characteristics of sleep apnea and neurodegeneration, as well as risk factors for subsequent neurodegeneration (5, 10, 19, 42, 46, 65). Clinical and basic science studies have reported sleep apnea contributes to the elevation of hypertension, inflammation, and oxidative stress (30, 49, 55, 64, 91, 94, 95). Indeed, hypertension, itself, is a primary risk factor for AD and PD and can exacerbate inflammation and oxidative stress through vascular dysfunction (10, 26, 42, 49, 65, 98, 105). In patients with PD, men are more likely than women to suffer from hypertension (96). While the incidence of hypertension in AD does not appear to be different between men and women, women with mid-life hypertension are more likely to be diagnosed with dementia later in life than men with the same condition (35). As women age, their risk of developing cognitive impairments increases if they have uncontrolled hypertension (61). Therefore, the association of hypertension with sleep apnea may not only increase a person's risk of subsequent

neurodegeneration, it also appears to be a determining factor in the type of neurodegeneration experienced in a sex-dependent fashion.

Men with sleep apnea are more frequently diagnosed with hypertension than women with sleep apnea (43). Typically, the hypertension is more severe in men than women, and their hypertension severity is positively associated with the severity of AHI (104). Alternatively, women with sleep apnea experience milder hypertension, which is sustained independent of AHI severity and often resistant to pharmacological therapeutics (104). In chronic intermittent hypoxia, an animal model of the hypoxic events experienced by patients with sleep apnea, sex differences in the manifestation of hypertension persist. Male rats are observed to experience an elevation in mean arterial pressure, similar to men with sleep apnea (55). However, in a study comparing male and female responses to chronic intermittent hypoxia, gonadally intact female rats did not experience the sustained hypertension observed in male rats. In fact, only estrogen-deficient ovariectomized female rats exhibited an elevation in mean arterial pressure (45). This indicates hormones play a crucial role in the impact of repetitive hypoxic events during sleep on vascular reactivity.

In addition to hypertension, the peripheral elevation in oxidative stress and inflammation documented in people with sleep apnea is integral to the mechanisms by which sleep apnea may preclude neurodegeneration. Children with sleep apnea have higher circulating inflammatory markers as well as elevated beta-amyloid expression, a marker often associated with cognitive impairment, than children without sleep apnea (38, 52, 53). Treatment of sleep apnea in those children appears to improve these conditions. In adults with sleep apnea (in the absence of other comorbid conditions), an increase in inflammatory biomarkers was observed in both men and women (12). Once again, sex differences were observed in the identity of which biomarkers were

more highly expressed. In another study that investigated only men, Kaczmarek, et al. reported an increase in endothelial nitric oxide synthase, hypoxia-inducible factor 1 alpha, and vascular endothelial growth factor, indicative of elevated oxidative stress within endothelial cells with sleep apnea (49). While anecdotal evidence exists to suggest a difference between men and women in sleep apnea induced vascular dysfunction and oxidative stress, that relationship remains to be definitively answered.

To further examine the impact of sleep apnea on physiology, investigators have used different animal models. A common animal model to examine the hypoxia associated with sleep apnea is chronic intermittent hypoxia, wherein room air is repetitively decreased to 8 - 10% of normal oxygen levels while the animal sleeps. Using this animal model, an increase in inflammation and oxidative stress markers in male rats exposed to chronic intermittent hypoxia has been observed (16, 73, 85, 88, 94, 95, 99). In our lab, increased oxidative stress and inflammatory dysregulation was observed in the periphery and the central nervous system, specifically within the entorhinal cortex and substantia nigra of male rats after only seven days exposure (95). Damage to these areas is implicated in the onset of AD and PD, respectively (14, 15). Chronic intermittent hypoxia models of longer duration and more severe hypoxic conditions induce inflammation and neuronal loss in the hippocampus of male rodents, which is indicative of advanced stage AD (36, 37, 72, 88, 99). This suggests the hypoxic events experienced during sleep apnea may be responsible for triggering oxidative and inflammatory events within brain regions responsible for cognition and motor control. Studies investigating the mechanisms of sleep apnea in female rodents are scarce and represent a need for further research. Interestingly, treatment of sleep apnea is correlated with a reduction of inflammatory biomarkers associated with AD (52, 64). In addition, treatment with anti-inflammatory agents appear to reduce the risk

for neurodegeneration compared to the general population (4, 52, 59, 62). It appears that addressing the underlying pathology of all stages of sleep apnea may be protective against the accumulation of neurodegenerative-inducing inflammation and oxidative stress.

Conclusion

Sleep apnea is a common disorder, which is underdiagnosed in most of the population. The observed sex differences in sleep apnea may be due to the actions of androgens and estrogens. The effects of sleep apnea go well beyond a lack of restfulness during the day to exacerbate the risk of cardiovascular and neurodegenerative diseases. It is possible sleep apnea contributes to neurodegeneration by inducing vascular dysfunction, resulting in elevation of oxidative stress and inflammation in a regional specific manner within the central nervous system. Preservation of vascular function may be influenced by sex-hormones. While the mechanisms of sleep apnea in cardiovascular disease have been highly investigated, its contributions to neurodegeneration are only recently beginning to be appreciated. Understanding how sleep apnea contributes to oxidative stress and inflammation within the central nervous system will provide valuable information in the search to treat neurodegeneration. Furthermore, investigation is needed into the contribution of sex and sex hormones to provide protection or exacerbation of risk for sleep apnea and its comorbidities.

Acknowledgements

This study was supported by the Texas Alzheimer's Research and Care Consortium (TARCC) funded by the state of Texas through the Texas Council on Alzheimer's Disease and Related Disorders. Research reported here was supported by the National Institutes of Health (NIH) R01 NS088514 to RLC and NIH training grant T32 AG 020494 to BDS.

FIGURE 1

FREQUENCY OF SLEEP DISORDERS IN TARCC PARTICIPANTS

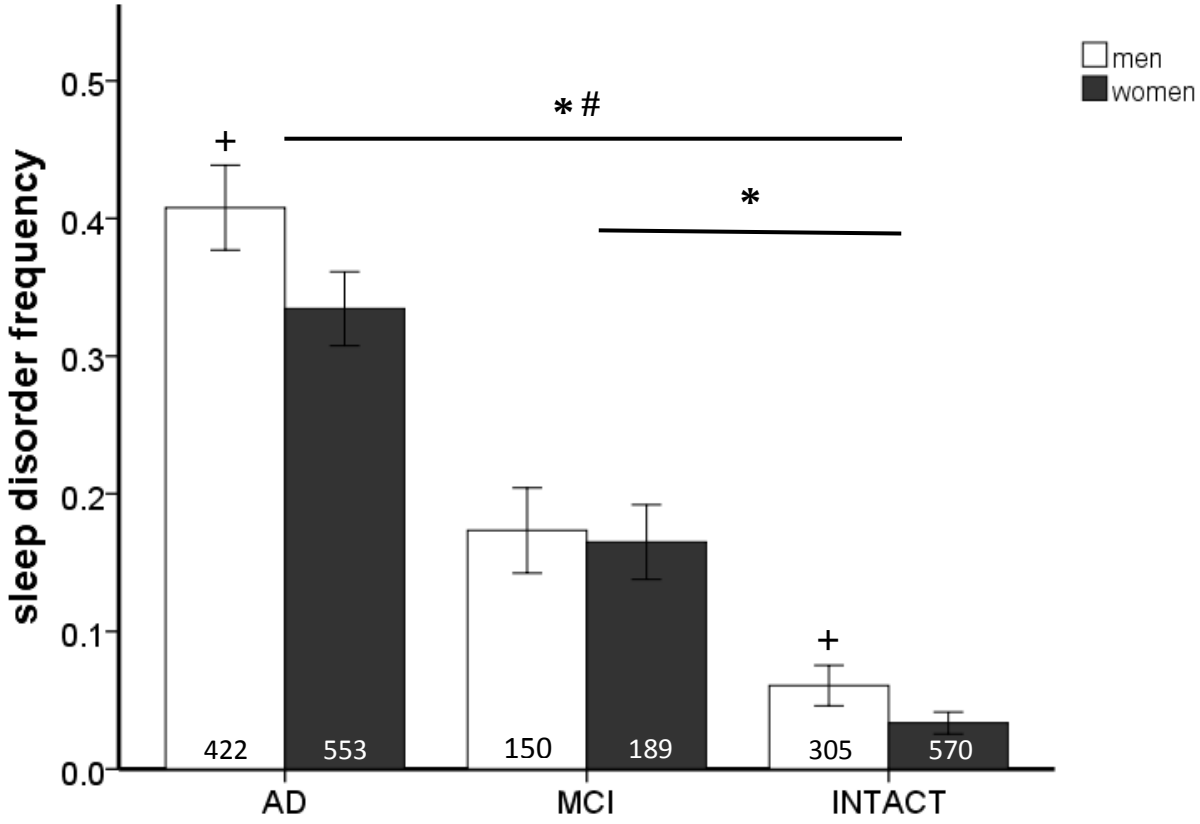

Figure 1: The frequency of sleep disorders was obtained by TARCC participant or caregiver answers on the Neuropsychiatric Inventory Questionnaire (51) (Table 1). Participants with cognitive impairment reported more sleep disturbances than cognitively intact participants (Intact). Additionally, participants with Alzheimer's disease (AD) reported more sleep disturbances than participants diagnosed with mild cognitive impairment (MCI) ($F(2,1667) = 130.172$, $p < 0.05$). Men reported more sleep disturbances than women ($F(1,1667) = 3.805$, $p < 0.05$). Analysis by ANOVA with Tukey post hoc testing. ; * versus INTACT; # versus MCI; + versus women ($p = 0.07$).

TABLE 1

POPULATION CHARACTERISTICS OF TARCC PARTICIPANTS

	Men			Women		
Variable	**N**	**Mean**	**St. Dev.**	**N**	**Mean**	**St. Dev.**
Age (years)	877	72.79	8.88	1312	72.21	9.67
	min. age		*max. age*	*min. age*		*max. age*
	50		94	50		102
	N	**%**		**N**	**%**	
hyperlipidemia	557	*63.51*		725	*55.26*	
hypertension	559	*63.74*		814	*62.39*	
obese	192	*21.89*		355	*24.92*	
Alzheimer's disease	422	*48.12*		553	*42.69*	
mild cognitive impairment	150	*17.10*		189	*12.42*	
cognitively intact	305	*34.78*		570	*44.65*	
< high school diploma	105	*11.97*		224	*12.05*	
high school diploma	131	*14.94*		364	*26.21*	
≤ 4 yrs. college	403	*45.95*		525	*43.00*	
> college	238	*27.14*		199	*18.75*	

Table 1: Characteristics of sample population used to determine frequency of sleep disturbances. Blood samples were provided by Caucasian men and women enrolled in the longitudinal research cohort of the Texas Alzheimer's Research Care and Consortium (TARCC). Normal controls performed within normal limits on all cognitive testing. MCI was defined using Petersen's criteria (75) and AD patients met consensus-based diagnosis for probable AD based on NINCDS-ADRDA criteria (66). Institutional Review Board approval was obtained at each TARCC site and written informed consent was obtained from participants and/or caregivers.

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CHRONIC INTERMITTENT HYPOXIA INDUCES OXIDATIVE STRESS AND INFLAMMATION IN BRAIN REGIONS ASSOCIATED WITH EARLY STAGE NEURODEGENERATION

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*_published in_ Physiological Reports, 2017. 5(9): p. e13258-n/a.

Abstract

Sleep apnea is a common comorbidity of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). Previous studies have shown an association between elevated oxidative stress and inflammation with severe sleep apnea. Elevated oxidative stress and inflammation are also hallmarks of neurodegenerative diseases. We show increased oxidative stress and inflammation in a manner consistent with early stages of neurodegenerative disease in an animal model of mild sleep apnea. Male rats were exposed to 7 days chronic intermittent hypoxia (CIH) for 8 hours per day during the light period. Following CIH, plasma was collected and tested for circulating oxidative stress and inflammatory markers associated with pro-inflammatory M1 or anti-inflammatory M2 profiles. Tissue punches from brain regions associated with different stages of neurodegenerative diseases (early-stage – substantia nigra and entorhinal cortex, intermediate – hippocampus, late-stage – rostral ventrolateral medulla and solitary tract nucleus) were also assayed for inflammatory markers. A subset of the samples was examined for 8-hydroxydeoxyguanosine (8-OHdG) expression, a marker of oxidative stress induced DNA damage. Our results showed increased circulating oxidative stress and inflammation. Further, brain regions associated with early-stage (but not late-stage) AD and PD expressed oxidative stress and inflammatory profiles consistent with reported observations in pre-clinical neurodegenerative disease populations. These results suggest mild CIH induces key features that are characteristic of early-stage neurodegenerative diseases and may be an effective model to investigate mechanisms contributing to oxidative stress and inflammation in those brain regions.

Sleep apnea is a comorbidity of several neurodegenerative diseases, such as Alzheimer's disease (AD) (6, 26, 73) and Parkinson's disease (PD) (3). Sleep apnea occurs 2-3 times more frequently in men than women, and the prevalence of sleep apnea increases with age (23, 80). Interestingly, in patients with PD who suffer from sleep disordered breathing, the severity of sleep apnea corresponds with the severity of PD (20). This suggests sleep apnea may contribute to the risk of neurodegeneration.

Sleep apnea is a common disorder resulting in interruptions in breathing that cause alternating periods of reduced oxygen inspiration (apnea or hypopnea) followed by rapid reoxygenation while a patient sleeps (18). The reduction in oxygen inspiration leads to hypoxia, hypercapnia, and decreased intrathoracic pressure (19). The periodic rapid reoxygenation events may contribute to the pathogenesis of sleep apnea (8, 37). The severity of sleep apnea is often determined by the apnea/hypopnea index (AHI), which measures the number of times per hour a hypoxic event occurs during sleep (66). The AHI classification of mild, moderate, or severe sleep apnea is 5, 15, and 30 events per hour, respectively.

Recent studies have shown hypoxic events can be either neuroprotective or neurotoxic depending on the severity, frequency, and duration of the hypoxia (8, 35, 39, 57, 64). Acute sustained hypoxic events, defined by occurrences lasting less than four hours, appear to precondition cells to subsequent insults by upregulating protective pathways within neurons (8, 55, 71). In contrast, chronic intermittent hypoxia (CIH), which occurs repeatedly during sleep every night, has been associated with increased neurodegeneration in a variety of brain nuclei (28, 29, 58, 78), which can impact different functions, such as cognition, motor ability, and homeostatic functions (15, 58, 70, 78). Increased oxidative stress and inflammation, key

characteristics of neurodegenerative diseases (5, 36, 61, 62), are observed in sleep apnea (43, 48).

Age-associated oxidative stress may be a major contributor to many age-related diseases. Elevated oxidative stress is implicated in the progression of neurodegeneration by leading to protein dysfunction and a resultant inflammatory environment within cells (5, 17, 52). Previous studies in our lab have demonstrated oxidative stress can induce neuroinflammation which, in turn, can elevate oxidative stress above a threshold which is neurotoxic to cells and leads to neuronal apoptosis (34) and is disrupted by inhibiting inflammatory pathway components.

One major component of the inflammatory system are macrophages. Within the central nervous system (CNS), macrophages and microglia are activated by cytokines to differentiate into either a pro-inflammatory (M1) or an anti-inflammatory (M2) profile (21, 41). Cytokines such as IL-6, IL-5, TNF-α, and IFN-γ are classically responsible for activation of microglia into the pro-inflammatory M1 profile to remove pathogens and damaged tissue, as well as activate apoptotic pathways. Alternatively, IL-4, IL-10, and IL-13 cytokines allow for microglial differentiation into anti-inflammatory M2 profiles to promote cell survival and inhibit inflammatory pathways (table 2). Activated microglia then release cytokines consistent with their profile to further induce additional immune responses. Under homeostatic conditions, these activated profiles are balanced to maintain cellular health (21). However, dysregulation of these complementary profiles can caused by chronic activation of the inflammatory system and is associated with detrimental effects (31, 32, 41, 59, 60, 81) in many disease states. An increase in pro-inflammatory M1 cytokines has been observed in neurodegeneration (21). These same M1 cytokines are also elevated in patients with sleep apnea (30, 48, 70) and in animal models (67,

70). Chronic activation of pro-inflammatory M1 markers associated with sleep apnea may be a contributing risk factor to neurodegenerative diseases (13, 47, 53).

The rodent model of CIH allows for the study of hypoxia-reoxygenation mechanisms, similar to those experienced by patients with sleep apnea (25). Models of severe CIH have been documented to mimic the hypoxia experienced by patients with severe sleep apnea (AHI > 30), as well as elevated inflammation associated with neurodegenerative diseases (28, 39, 70). However, few studies have examined the impact of mild hypoxia (AHI < 30) on inflammation or oxidative stress within specific regions of the brain. We hypothesize mild chronic intermittent hypoxia increases oxidative stress and inflammation in brain regions affected by early stage neurodegenerative diseases.

To examine the role of sleep apnea on oxidative stress and neuroinflammation in brain areas at risk for neurodegeneration, we exposed male rats to CIH (AHI = 10), a model of mild sleep apnea. Five brain nuclei that have been shown to be affected by neurodegenerative diseases at different stages of progression were selected and assessed for changes in oxidative stress and M1/M2 activating cytokine expression. Brain nuclei known to be affected during early-stage neurodegeneration are the substantia nigra (SN) in PD (12, 14) and the entorhinal cortex (ETC) in AD (9, 49). Neurodegeneration is observed in the hippocampus during intermediate stages and is believed to be a leading contributor to the dementia experienced by patients with AD (63). The rostral ventrolateral medulla (RVLM) and the solitary tract nucleus (NTS) are affected during advanced late-stage neurodegenerative diseases, and play a major role in maintaining homeostatic functions (1, 15, 50, 77). Our results show significant elevations in oxidative stress and pro-inflammatory M1 cytokines in the SN and ETC, regions associated with early-stage

neurodegeneration. This is the first study we are aware of to characterize the inflammatory profile of the SN and brainstem regions in response to mild CIH.

Methods

Animals: All experiments were conducted according to National Institute of Health guidelines on laboratory animals and approved by the Institutional Care and Use Committee at UNT Health Science Center. 37 adult male Sprague-Dawley rats (250-300 g body weight, Charles River) were individually housed in a temperature controlled environment with the lights on a 12:12 hour cycle. Food and water were provided *ad libitum*. Male rats were used because sleep apnea is more prevalent in men than women (44).

Chronic Intermittent Hypoxia (CIH): One week after arrival, rats were separated into either normoxia (n = 13) or CIH (n = 18) treatment groups. Rats were placed into custom-built Plexi-glass chambers to acclimate to the CIH apparatus for one week at normoxic conditions (21% oxygen), as previously described (15). Acclimation to the chambers was followed by CIH exposure for 7 days from 8 am to 4 pm during the light (sleep) cycle. Oxygen concentrations were controlled by timers, which alternate the flow of room air and nitrogen into the chambers. Our CIH protocol involves 6 minute cycles of low oxygen (10%) followed by reoxygenation (21%) for 8 hours during the light phase to model an AHI of 10 as previously described (40). This protocol is used to model the frequency of hypoxic episode experienced by patients with mild sleep apnea (24). Specifically, nitrogen is injected into the chamber for 90 seconds and is titrated so that 10% oxygen occurs within each chamber a few seconds after nitrogen injection halts. Over a period of 90 seconds, no gas is injected and the oxygen concentration begins to slowly rise. Room air is then infused into the chamber for a total of 90 seconds, which returns the

oxygen levels to 21% within 30 seconds. Normal room oxygen levels are then maintained for an additional 90 seconds. This cycle repeats throughout the 8 hour treatment time. For the remaining 16 hours, animals were exposed to room air. To control for sleep deprivation due to noises from the CIH apparatus, normoxic controls were housed under similar conditions but were not exposed to hypoxia.

Sample Collection: Within 16 hours of the final CIH exposure, during the first two hours of the light phase, animals were deeply anesthetized (inactin, 100 mg/kg ip) and sacrificed as previously described (40). Blood was collected in 7 mL EDTA tubes. The samples were then centrifuged at 2,240 x g for 10 min at 4°C. Plasma was removed and stored in microcentrifuge tubes at -80°C until assayed.

Brains were immediately removed upon euthanasia, flash frozen in PBS on dry ice, and then sliced into 1 mm coronal sections using a commercially available matrix (ASI Instruments, Warren MI). Brain nuclei containing the rostral ventrolateral medulla (RVLM) (-11.80 mm from Bregma), solitary tract nucleus (NTS) (-13.68 mm from Bregma), substantia nigra (SN) (-5.30 mm from Bregma) or entorhinal cortex (ETC) (-5.30 mm from Bregma), and dorsal hippocampus (-4.52 mm from Bregma) were isolated according to Paxinos and Watson's The Rat Brain Stereotaxic Coordinates (56) using blunt 20 gauge needles attached to 1 mL syringes, immediately frozen on dry ice in 1.5 mL microcentrifuge tubes, then stored at -80° C until homogenized for assays.

Tissue Homogenization: Each sample was incubated in 50 μL RIPA lysis buffer (Amresco) with 3 μM phosphatase inhibitor (Sigma-Aldrich) and 1 μM dithiothreitol (Sigma-Aldrich) then incubated on ice prior to sonication three times. Samples were then centrifuged 20 minutes at 12,000 x g at 4° C. The supernatant was extracted and transferred to a clean 1.7 mL

microcentrifuge tube. Protein quantification was assessed by using the Modified Lowry Protein Assay Kit (Thermo Scientific) and homogenate was stored at -80°C until used in multiplexing protocols.

Advanced Oxidative Protein Products (AOPP) assay: Circulating oxidative stress was assayed using Cell Biolabs, Inc. OxiSelect Advanced Oxidative Protein Products assay kit, according to our previously published protocol (16). This kit measures the amount (uM) of all oxidized proteins in the sample relative to a known standard. Chloramine in the kit reacts with oxidized proteins to produce a color change which can be read at 340nm.

Inflammatory Panel: IL-6, TNF-α, IFN-γ, and IL-5, protein levels were used as indicators of pro-inflammatory M1 macrophage recruitment, while IL-10, IL-13, and IL-4 protein levels were used as indicators of anti-inflammatory M2 macrophage activation. Cytokine levels in brain tissue homogenate samples were quantified using Proinflammatory Panel 1 (rat) V-PLEX Kit from Meso Scale Diagnostics (**tables 2-4**). This immunoassay allows for quantification (pg/ml) of multiple analytes from a single sample using a sandwich ELISA protocol with each analyte of interest located in a specific region of each well. 120 μg of each tissue sample was quantified using the V-PLEX assay. Plasma cytokine levels were measured using Biorad's Bioplex Rat Th1/Th2 12-Plex kit and fluorescence measured on a Luminex platform (MAGPIX, Luminex Corporation, Austin, TX). Plasma samples (diluted 1:4) were loaded into each well of a 96 well pate and capture antibodies attached to magnetic beads reacted with each analyte of interest within each sample on a different region of the bead. This immunoassay quantifies the amount (pg/ml) of each cytokine relative to a known standard using Bio-Plex Manager Software 6.1. Quantities of each cytokine were normalized to normoxic controls. To analyze the effects of CIH on pro-inflammatory M1 (IL-5, IL-6, TNF-α, & IFN-γ) and anti-inflammatory M2 (IL-4, IL-10,

& IL-13) cytokines as a group, individual cytokine values were summed together to create a composite value.

Immunohistochemistry A subset of rats exposed to either CIH (n = 3) or normoxic (n = 3) conditions were transcardially flushed with 0.1 M PBS, and then perfused with 4% paraformaldehyde in 0.1 M PBS following 7 days CIH exposure. Brains were removed from the skull and stored overnight in 4% paraformaldehyde at 4°C. 24 hours later, brains were transferred to 30% sucrose in PBS at 4°C for 3 days, then sliced on a cryostat (Thermo Scientific, CryoStar NX70) into 40 μm coronal sections containing the regions of interest as previously described (15). Three separate sets of coronal sections containing the SN, ETC and dorsal hippocampus (-4.80 to -6.04 mm from Bregma), RVLM (-11.30 to -12.72 mm from Bregma), or NTS (-13.68 to -14.08 mm from Bregma) were collected from each brain and stored in cryoprotectant at -20°C until they were processed for immunofluorescence (76).

One set of sections from each animal was rinsed in PBS and incubated in PBS in 3% horse serum for 4h. The sections were rinsed and incubated for 72 hours at 4°C in primary goat 8-hydroxydeoxyguanosine (8-OHdG) antibody (Abcam ab10802, 1:10,000). Afterward, the sections were rinsed in PBS and incubated in secondary antibody (Alexa Fluor 488 Goat anti-Rabbit 1:2000; Abcam ab150105) overnight at 4°C. Sections were then rinsed in PBS, mounted on slides, and sealed using Vectashield mounting medium containing DAPI. Slides were stored at 4°C until imaged.

Images of DAPI and 8-OHdG immunofluorescence were captured from each section using an inverted fluorescence microscope (VWR International) equipped with a digital camera (Coolsnap MYO, Photometrics, Tuscon, AZ). The numbers of 8-OHdG/DAPI+ cells per image

were quantified using NIS Elements Imaging Software 4.50 (Nikon Instruments, Inc, Melville, NY).

Statistical analysis: Assay results are reported as percent of control (individual value / (average of control values) x 100). IBM SPSS (SPSS v. 23, IBM, 2015) was used for statistical analysis. Independent *t*-tests assessed the difference between the means of AOPP, cytokines, and 8-OHdG expression by treatment. Pairwise *t*-tests were used to investigate the difference between M1 and M2 cytokine expression within animals exposed to CIH. Results are shown as mean ± SEM. Statistical significance was at $p < 0.05$.

Results

Exposure to CIH increases circulating oxidative stress

One hallmark of neurodegenerative diseases is elevated oxidative stress (5). Circulating AOPP levels were measured as a marker of oxidative stress. Plasma from animals exposed to CIH exhibited an average of 24% more oxidative stress than control animals, which was statically significant (Figure 2; $t(28) = -2.481$; $p < 0.05$). These results show that mild CIH increases systemic oxidative stress, consistent with the elevation in oxidative stress observed in patients with neurodegenerative diseases (17).

Exposure to CIH increases oxidative stress in brain regions associated with neurodegenerative diseases

To examine if CIH induces oxidative stress in the brain, we quantified 8-OHdG protein expression. 8-OHdG is a marker of oxidative stress induced DNA damage. CIH was associated with significant oxidative stress mediated DNA damage in the SN, layer II of the ETC, and

dorsal hippocampus, brain regions linked with PD and AD, respectively (Figures 2A and 4A). The number of cells with oxidative stress induced DNA damage following CIH exposure were significantly greater in the SN ($t = -4.436$; $p < 0.05$), the ETC ($t = -2.796$; $p < 0.05$), the dentate gyrus ($t = -11.620$; $p < 0.05$), and the CA1 regions of the hippocampus ($t = -2.515$; $p < 0.05$) (Figures 2B and 4B). No 8-OHdG immunoreactivity was observed in the RVLM or NTS (data not shown), indicating that mild CIH does not increase oxidative stress induced DNA damage to brain regions affected during late-stage neurodegenerative diseases. This evidence supports our hypothesis that mild CIH elevates oxidative stress in brain regions linked with early to intermediate stage neurodegeneration, but not late-stage neurodegeneration.

Alterations in the M1/M2 cytokine profiles are observed in animals exposed to CIH

CIH induced a mild peripheral inflammatory response (Figure 5) similar to the elevated inflammation observed in patients and animal models of advanced neurodegenerative diseases (13, 61, 62, 82). Both M1 ($t (31) = -2.431$; $p < 0.05$) and M2 ($t (31) = -2.456$; $p < 0.05$) circulating cytokines were significantly elevated after CIH compared to control rats under normoxic conditions.

Interestingly, inflammatory profiles in the CNS were altered in a brain region specific manner. Differences in cytokine expression due to CIH were observed several regions (Figure 6, tables 2-4). In the SN, an area associated with early-stage PD, pro-inflammatory M1 cytokines, but not anti-inflammatory M2 cytokines, were significantly elevated in animals exposed to CIH (Figure 6A; $t (25) = -3.076$; $p < 0.05$, table 3). In the RVLM, which is implicated in later stages of neurodegenerative diseases, CIH was associated with significant decreases in both M1 and M2

cytokines compared to normoxic controls (Figure 6B; M1: $t(26) = 2.055$; M2: $t(26) = 2.600$; both $p < 0.05$, table 5).

A different pattern was observed in regions affected by AD. In the ETC, a region associated with early stage AD, pro-inflammatory M1 cytokines were significantly elevated over anti-inflammatory M2 cytokines within each animal exposed to CIH (Figure 6C; $t(8) = 2.379$, $p < 0.05$, table 3). Within the dorsal hippocampus, M2 cytokines were significantly elevated compared to M1 cytokines within animals exposed to CIH (Figure 6D; $t(14) = -2.337$; $p < 0.05$, table 4). No differences were observed in the NTS (data not shown).

Discussion

Mild CIH induces effects in brain regions consistent with early to intermediate stage neurodegenerative disease. Patients with preclinical AD exhibit elevated systemic oxidative stress and inflammation (51, 61). Pathological protein aggregates associated with AD appear in the second layer of the ETC during early stages (11, 38), and then progress to the hippocampus during intermediate stages when AD symptoms begin to manifest (9, 10, 49). During later stages, those effects spread to cortical and brainstem areas, and are associated with a higher severity of symptoms as well as a loss of autonomic functions (9, 10). Similarly, patients with early-stage PD exhibit elevated systemic oxidative stress, inflammation, and protein aggregates in the SN (12, 51, 62). These systemic effects have been observed in rodent models of severe CIH (30, 43, 48). Severe sleep apnea has been established as a comorbidity of several neurodegenerative diseases (2, 3) and is associated with elevated oxidative stress and inflammation. Recent studies (68, 79) indicate men with sleep apnea are at higher risk to develop PD and the severity of sleep apnea increases with the severity of PD (20). This suggests sleep apnea may be one contributor

to the risk of developing a neurodegenerative disease. While clinical treatment of patients with moderate/severe sleep apnea is considered standard, treatment of mild sleep apnea is optional (24, 42). Our results with mild CIH suggest that mild sleep apnea may be a risk factor for neurodegeneration.

Our results show mild CIH elevates oxidative stress both peripherally and centrally in a pattern consistent with early neurodegenerative diseases (51). Further, this effect is not contributable to sleep deprivation as it is not observed in the normoxic controls. Oxidative stress has been implicated in AD and PD progression, with detrimental effects observed in the SN, ETC, and hippocampus during the early and intermediate stages of these diseases (5, 51). The number of cells in these regions exhibiting DNA damage due to oxidative stress was elevated in our model of mild CIH (Figure 3 & 4). This may be a precursor to the hippocampal damage observed in longer and more severe models of CIH (28, 29, 67, 78). No oxidative stress-induced DNA damage was observed in areas associated with late-stage neurodegenerative disease symptoms. It is conceivable that longer exposure to mild CIH may contribute to an environment in which damage to later stage regions occurs.

Our results also demonstrate inflammation consistent with early-stage neurodegenerative diseases. An overall increase in circulating inflammatory markers is observed in animals exposed to CIH (Figure 5) similar to the rise in inflammatory markers observed in patients with neurodegenerative diseases (4, 21, 59). Prior studies have shown specific brain regions exhibit different inflammatory responses to models of intermittent hypoxia. These responses are dependent upon the severity of the model and whether the exposure to intermittent hypoxia is chronic or acute. (29, 65, 70).

In our model of CIH, we also observed regionally different responses in inflammatory markers (Figure 6) consistent with previously published observations (67, 70). Microglial differentiation is complex and guided by a variety of signals dependent upon the immediate needs of the specific region. Expression of either M1 pro-inflammatory markers or M2 anti-inflammatory markers is in response to the cytokines present within local environmental milieu (table 2). Cytokines may be released by astrocytes or neurons in addition to other activated microglia. These cytokine signals further activate naïve microglia to respond to the needs of the cell and differentiate into either pro-inflammatory M1 or anti-inflammatory M2 microglia primed to either remove damage or promote neuronal survival (74). The M1/M2 cytokine profiles observed in this study are consistent with previously published data observed in cases of early-stage neurodegenerative disease (21, 46).

We observed a pro-inflammatory profile in the SN and ETC. In the SN and the ETC, an elevation of M1 pro-inflammatory cytokines was observed in animals exposed to CIH. This suggests the local environment in each of these early stage regions is favorable to elevated M1 microglial activation and mimics the microglial response in those regions in patients with either PD (27) or early-stage AD (38, 46). While these initial changes are mildly elevated, an increase in inflammation can further increase oxidative stress. This may be one mechanism whereby CIH switches from a neuroprotective environment to a neurotoxic one.

Interestingly, we observed an increase in the anti-inflammatory cytokine profile in the hippocampus, which is consistent with results seen in patients during the early to intermediate stages of AD (74). This may reflect early compensatory mechanisms by microglia to attempt to protect against further damage. The overall decrease in both M1 and M2 profiles in the RVLM may also be compensatory to suppress early damage or it could be indicating that there is no

inflammatory response, as no oxidative stress damage was observed in this region. No significant effects in the M1/M2 profiles were observed in the NTS. The results observed in these two regions may be due to processes which maintain homeostatic function. These results are consistent with the literature implicating these areas in late-stage AD (9).

Oxidative stress damage in layer II of the ETC is one of the earliest observable hallmarks in AD pathology, and it occurs during stages I/II prior to clinical symptom manifestation (11, 72). In PD, oxidative stress levels are elevated systemically and in the CNS during pre-clinical stages (51, 54, 62). Mild CIH elevates oxidative stress both in the periphery and in brain regions associated with each of these diseases in a manner similar to early stage observations. Current efforts towards developing a biomarker panel to identify individuals during early-stages of neurodegenerative disease are ongoing (22, 45). Our data along with other publications indicate that oxidative stress should be considered for inclusion in these biomarker assays (7, 33, 75).

Currently, AD and PD are not able to be diagnosed during early stages of neurodegeneration. Treatments are typically initiated once a significant amount of neurodegeneration has occurred and do not prevent progression of these types of diseases. For example, clinical AD symptoms do not appear until Braak stage V when the pathology has progressed into the hippocampus and a significant amount of neuronal loss has occurred (10). PD is not diagnosed until there has been a loss of 80% of the dopaminergic cells within the SN (12, 44). Therefore, efforts have been focused toward identifying early modifiable factors to prevent neuronal loss and improve therapeutic outcomes. Our results indicate that mild CIH may be one modifiable factor and an appropriate model to study mechanisms leading to elevated oxidative stress and inflammation in early-stage neurodegenerative disease associated brain regions.

While our initial observations are promising, there are several limitations to our study, such as a lack of investigation into downstream inflammatory signaling, neurodegenerative markers, and behavioral indexes. Therefore, future studies will target motor and cognitive behaviors, downstream markers of pro-inflammatory activation (i.e. cyclooxygenase-2 protein expression), and traditional biomarkers of neurodegenerative diseases, such as Lewy body or tau protein accumulation.

Acknowledgements

We would like to thank Joel T. Little and William Davis for their technical assistance.

Funding

This study was supported by the Alzheimer's Association New Investigator Research Grant NIRG-14-321722, NIH R01 NS088514, and a UNTHSC Seed Grant to R. L. Cunningham, P01 HL088052 Grant to J. T. Cunningham, and NIH T32 AG 020494 to B. Snyder.

FIGURE 2

CHRONIC INTERMITTENT HYPOXIA INDUCES OXIDATIVE STRESS

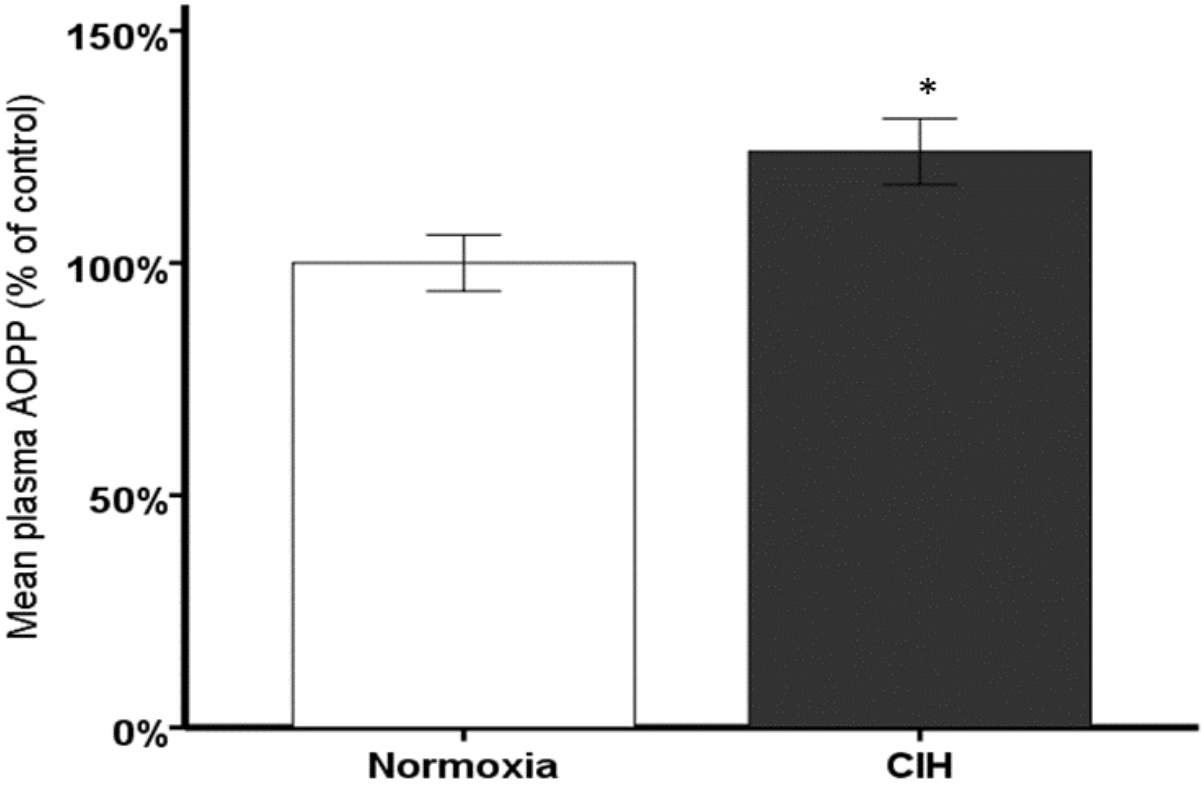

Figure 2 Plasma AOPP levels of animals exposed to CIH: 7 day exposure to CIH (n = 18) significantly increases circulating oxidative stress in male rats. * $p \leq 0.05$

Figure 3 Significantly elevated expression of 8-OHdG was observed in brain regions associated with early-stage neurodegenerative diseases following 7 days of CIH exposure. $* p \leq 0.05$ **(A)** An elevation of oxidative stress in animals exposed to 7 days CIH is observed in SN and layer II of the ETC as compared to controls animals. **(B)** CIH increased oxidative stress in the SN and ETC layer II. *Data presented as mean number of cells exhibiting 8-OHdG immunoreactivity/ section ± s.e.m. Scale bar = 100 μm.*

FIGURE 3

CHRONIC INTRMITTENT HYPOXIA INCREASES OXIDATIVE STRESS IN SUBSTANTIA NIGRA AND ENTORHINAL CORTEX

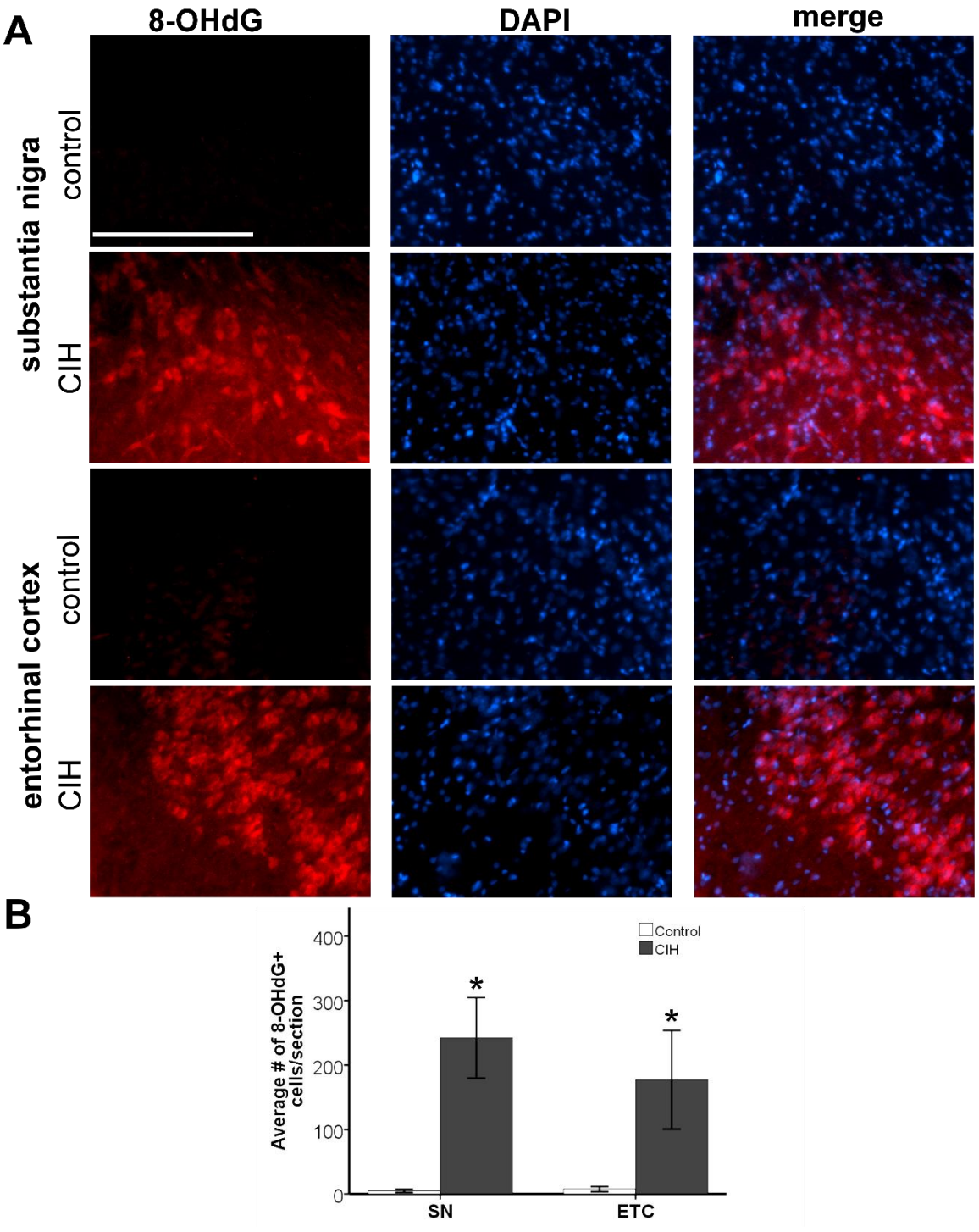

Figure 4 Significantly elevated expression of 8-OHdG was observed in brain regions associated with intermediate AD following 7 days of CIH exposure. * $p \leq 0.05$ **(A)** An elevation of oxidative stress in animals exposed to 7 days CIH is observed in both the dentate gyrus and the CA1 regions of the hippocampus. **(B)** CIH increased oxidative stress in the CA1 and dentate gyrus (DG). *Data presented as mean number of cells exhibiting 8-OHdG immunoreactivity/ section ± s.e.m. Scale bar = 100μm.*

FIGURE 4

CHRONIC INTERMITTENT HYPOXIA INCREASES OXIDATIVE STRESS IN HIPPOCAMPAL CA1 AND DENTATE GYRUS REGIONS

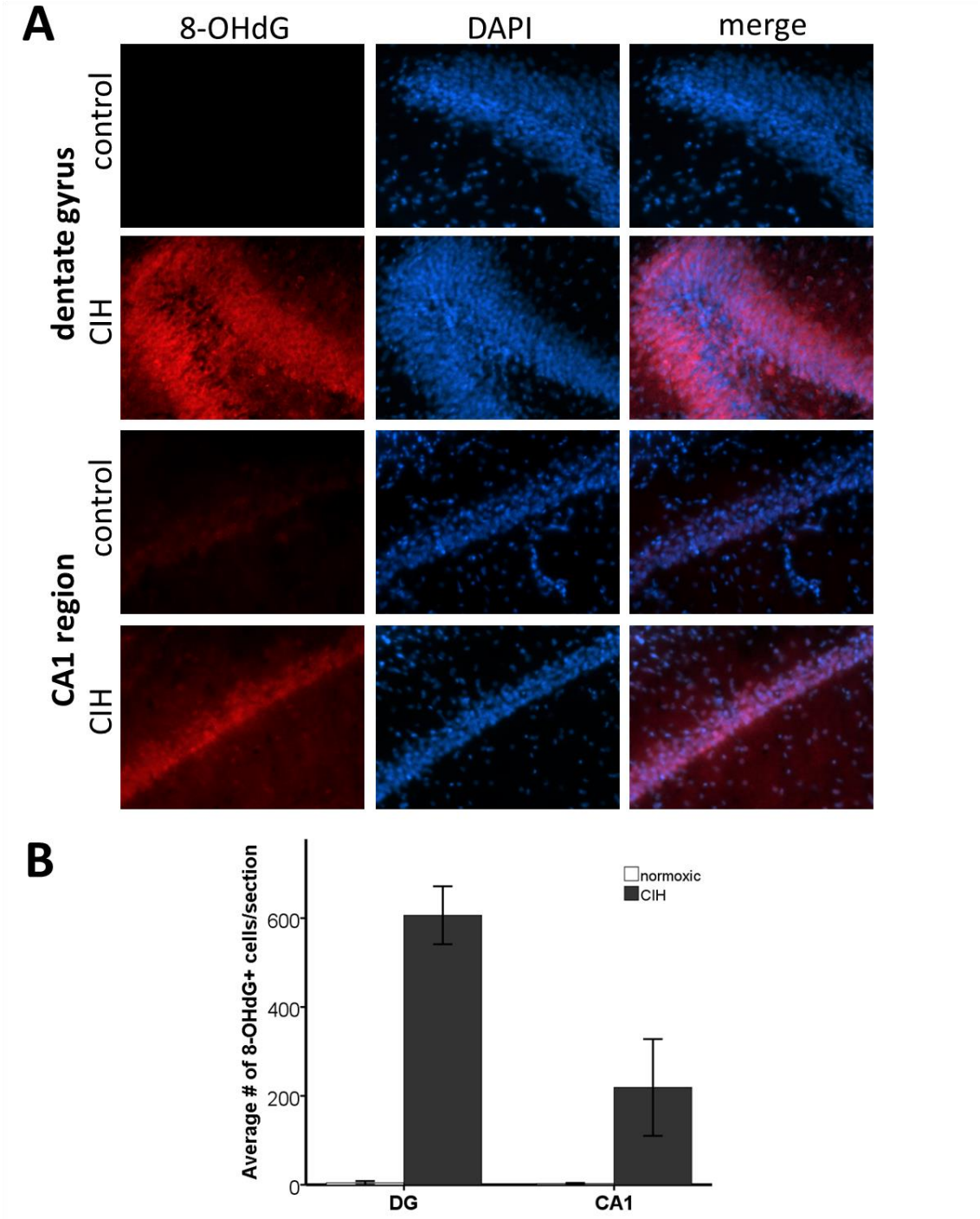

FIGURE 5

CHRONIC INTERMITTENT HYPOXIA INCREASES PLASMA INFLAMMATION

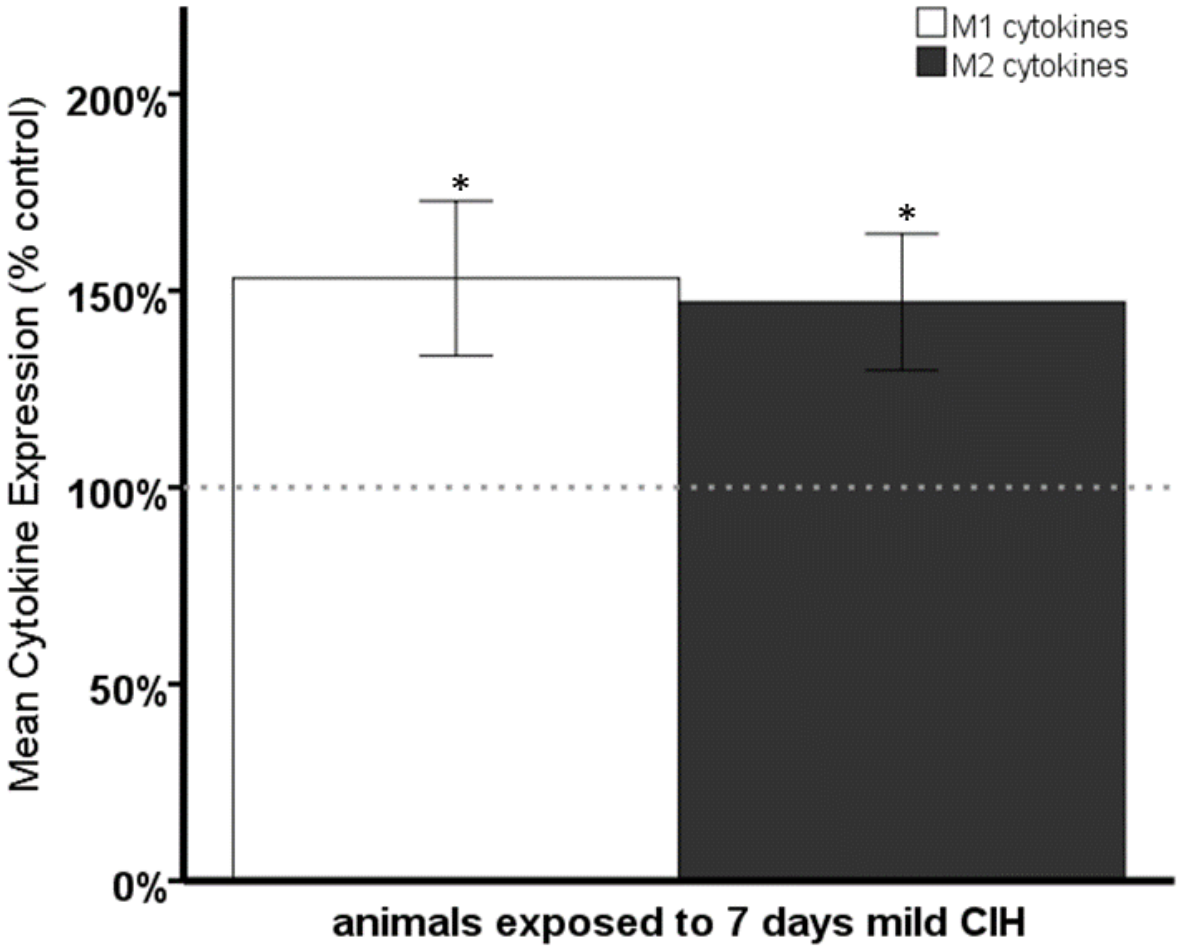

Figure 5 CIH induces an increase of circulating M1 (IL-6, TNF-α, IFN-γ, IL-5) and M2 (IL-4, IL-10, IL-13) inflammatory markers. Dotted line indicates normalized controls. (normoxia = 13, CIH = 18) * $p \leq 0.05$

Figure 6 Region specific inflammatory responses to CIH are observed in the brain, resulting in a pro-inflammatory profile in the SN and ETC. An anti-inflammatory profile is observed in the dorsal hippocampus. *Dotted line indicates normalized controls. * significant compared to control, # significant difference within animal* **(A)** A significant elevation of M1 cytokines in the SN of animals exposed to CIH leads to a higher M1 cytokine profile than M2 profile in that brain region. (normoxia = 12, CIH = 15) **(B)** An overall reduction of M1 and M2 cytokines is observed in the RVLM of animals exposed to CIH. No significant difference in the M1/M2 cytokine profile was observed in this region associated with late-stage neurodegenerative diseases. (normoxia = 13, CIH = 15) **(C)** M2 cytokines are significantly lower than M1 cytokines in the ETC of animals exposed to CIH, which elevates the M1profile over the M2 profile. (normoxia = 11, CIH = 9) **(D)** M1 cytokines are significantly lower than M2 cytokines in the HIPP following CIH. (normoxia = 13, CIH = 15)

FIGURE 6

CENTRAL NERVOUS SYSTEM INFLAMMATORY RESPONSE TO CHRONIC INTERMITTENT HYPOXIA

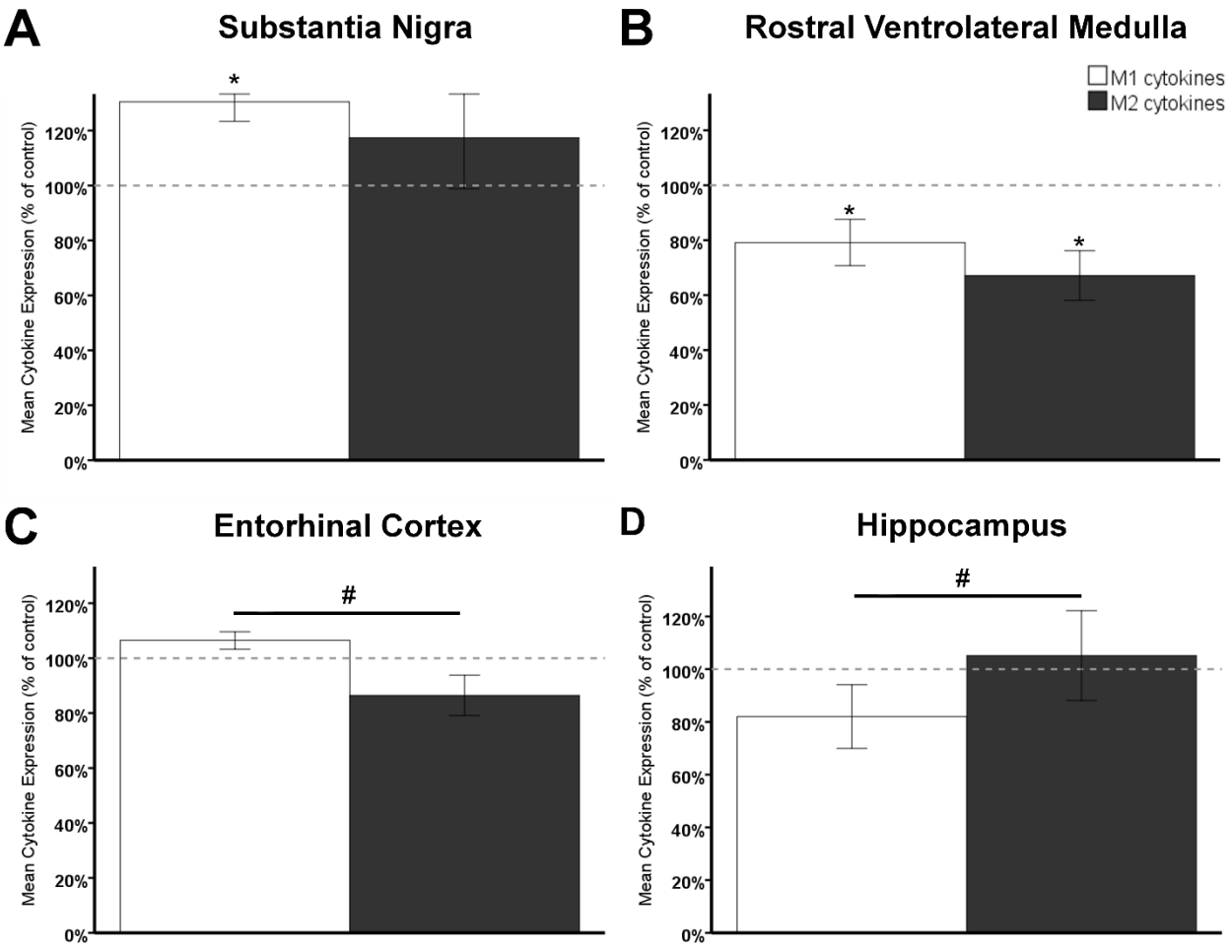

TABLE 2

CYTOKINES OF INTEREST

Microglia profile	**Cytokine**	**Physiological effects**	**Reference**
M1	IL-6 TNF-α IFN-γ	pro-inflammatory pathogen removal apoptotic events	*Baumberger, et al., 2002 (4)* *Perry, et al., 2014 (59)* *Sierra, et al. 2007 (69)*
M2	IL-10 IL-4 IL-13	anti-inflammatory pro-survival signals wound healing	*Varnum, et al., 2012 (74)* *Du, et al., 2016 (21)*

Table 2 Cytokines of interest for this study and known role in macrophage recruitment. IL = interleukin, TNF = tumor necrosis factor, IFN = interferon

TABLE 3

CYTOKINE EXPRESSION IN THE SUBSTANTIA NIGRA AND ENTORHINAL CORTEX

	Substantia nigra		**Entorhinal cortex**	
cytokine	CIH (n=8)	p-value	CIH (n=16)	p-value
IL-10	110.63 ± 15.17	0.592	81.75 ± 10.99	0.240
IL-13	102.52 ± 16.22	0.910	102.27 ± 7.11	0.878
IL-4	142.27 ± 55.80	0.525	93.42 ± 13.45	0.797
IL-6	154.78 ± 14.99	0.023*	116.01 ± 8.49	0.217
TNF-α	148.73 ± 18.44	0.052*	93.66 ± 10.20	0.697
IFN-γ	105.61 ± 7.11	0.569	103.70 ± 5.53	0.691
IL-5	92.80 ± 13.73	0.635	114.81 ± 14.29	0.472

Table 3 Cytokines per region implicated in early stage neurodegeneration. *Values are expressed as percent of control (pg/ml) ± SEM. *= $p<0.05$.* IL = interleukin, TNF = tumor necrosis factor, IFN = interferon

TABLE 4

CYTOKINE EXPRESSION IN THE HIPPOCAMPUS

	Hippocampus	
Cytokine	CIH (n=16)	p-value
IL-10	102.88 ± 18.81	0.919
IL-13	99.00 ± 19.90	0.784
IL-4	100.13 ± 23.10	0.621
IL-6	85.08 ± 11.47	0.228
TNF-α	87.62 ± 16.46	0.997
IFN-γ	89.16 ± 11.44	0.316
IL-5	103.43 ± 7.19	0.814

Table 4 Cytokines per region implicated in intermediate stage neurodegeneration. *Values are expressed as percent of control (pg/ml) ± SEM. *= $p<0.05$.* IL = interleukin, TNF = tumor necrosis factor, IFN = interferon

TABLE 5

CYTOKINE EXPRESSION IN BRAINSTEM AREAS

	Solitary tract nucleus		**Rostroventrolateral medulla**	
Cytokine	CIH (n=8)	p-value	CIH (n=16)	p-value
IL-10	58.60 ± 14.97	0.171	70.96 ± 9.77	0.051*
IL-13	51.63 ± 13.96	0.404	75.45 ± 12.97	0.163
IL-4	63.13 ± 15.20	0.292	54.96 ± 10.93	0.005*
IL-6	70.29 ± 16.54	0.411	75.62 ± 9.05	0.037*
TNF-α	65.09 ± 17.17	0.226	69.93 ± 9.53	0.043*
IFN-γ	74.67 ± 9.72	0.106	73.22 ± 7.01	0.019*
IL-5	71.80 ± 13.69	0.344	77.04 ± 16.47	0.464

Table 5 Cytokines per region implicated in late stage neurodegeneration. *Values are expressed as percent of control (pg/ml) ± SEM. *= $p<0.05$.* IL = interleukin, TNF = tumor necrosis factor, IFN = interferon

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ANDROGEN ACTIONS

It is necessary to understand the metabolism of the steroid hormone, testosterone, to appreciate its role in behavior. In the periphery, testosterone is primarily synthesized from cholesterol by the gonads, with small amounts originating from the adrenal cortex (21). Although small amounts can also be synthesized in the brain (20), the hydrophobic nature of steroid hormones allows it to easily pass across the blood brain barrier to enter the CNS as needed. Within the brain, testosterone and its metabolites can have several actions. Testosterone's primary action is through activation of androgen receptors, which classically dimerize upon agonist binding and enter the nucleus of a cell to enact genomic changes (21). Genomic actions are typically long-term, and their effects take hours – days to observe. Non-genomic actions of testosterone are observable within minutes and are dependent upon calcium signaling (5, 11, 17). Rapid effects are observed even when testosterone is conjugated to BSA, a large protein which prevents testosterone from being able to enter the cell. This implicates the presence of a receptor for testosterone at the cell membrane (5, 7, 17, 19). Studies attempting to isolate and characterize this receptor are ongoing, and promise to provide valuable insight into testosterone's actions.

In brain regions where the enzyme aromatase is present, testosterone can be aromatized into the estrogen, estradiol (E2) (21). E2 is an agonist for estrogen receptors. Testosterone can also be reduced by 5α-reductase to dihydrotestosterone (DHT). DHT is a potent androgen receptor agonist, and has been used in research studies to elucidate the actions of testosterone at the androgen receptor. Recent studies have revealed that DHT can be further reduced to 3β-diol,

an estrogen receptor agonist, in the presence of the enzyme 3β-hydroxysteroid dehydrogenase (10). Both testosterone and E2 have been observed to increase hippocampal synaptogensis and spatial memory in a similar manner (1, 13). Treatment with either aromatase inhibitors or DHT inhibit these actions (2). Similarly, DHT can increase oxidative stress in midbrain dopaminergic neurons (11, 12).

There are several different animal models to choose from when investigating the contributions of testosterone. One commonly used model is the genetic androgen receptor knockout in rats (23). This allows for elimination of the androgenic pathway from birth. While a knockdown model may be appropriate for developmental studies, the loss of androgen signaling from birth has additional physiological consequences. Adult XY rats of this strain are phenotypically female, and estrogenic signaling pathways are also affected in this model. Since androgens play a crucial role in memory and physiological development, androgen insensitivity could result in physiological adaptations in the adult animal that would confound the outcome of investigations into age-related damage.

Several techniques exist to achieve removal or inhibition of a specific gene after normal development has occurred. These include transgenic conditional knockdown models, such as the cre-lox system, genome editing, such as CRISPR-Cas9, and chemogenetic tools, such as designer receptors exclusively activated by designer drugs (DREADDs) (8, 16, 18). Unfortunately, the transgenic androgen receptor knockdown models only exist in mice, which precludes it from rat studies. Furthermore, the use of tetracycline in the cre-lox system can affect inflammation, which is an important component in sleep apnea and neurodegenerative diseases. Tamoxifen can also be used to target the gene of interest, but, as an estrogen receptor inhibitor, may also affect the outcome of testosterone studies. Newer techniques such as CRISPR-Cas9 and DREADDs may

provide more flexibility, but are expensive and have not yet been utilized in hormone receptor studies. Although the use of DREADDs to examine the excitatory action of G-protein coupled receptors (18) in the presence of androgens may eventually provide valuable mechanistic information, off-target excitation by DREADDs which can affect behavioral outcomes is a concern (3). These mechanisms should be further defined prior to use in hormone studies.

Receptor antagonists can be used to pharmacologically verify specific pathway involvement. Existing androgen receptor antagonists block genomic signaling to varying degrees, but are not effective at inhibiting oxidative stress and inflammatory aspects of androgen rapid signaling pathways (11). Unpublished *in vitro* studies in our lab have demonstrated inhibition of classical androgen receptor signaling by flutamide or selective androgen receptor modulators does not block testosterone's generation of oxidative stress. *In vivo* studies suggest flutamide only partially blocks some aspects of androgen signaling (14), and in some cases, can even mimic androgen effects in the hippocampus (15). These considerations render current androgen receptor antagonist options undesirable for this current investigation into mechanisms which may contribute to neurodegeneration through oxidative stress generation.

Rather than manipulating receptor actions, sex hormones themselves can be controlled by surgical options. Gonadectomy, removal of the primary sex organs, allows for elimination of the sex hormone production at a specific point in time. This permits development to continue normally throughout adolescence prior to the manipulation of hormones. Several methods are widely used to replace circulating hormones or their metabolites. Daily i.p. injections mimic the diurnal fluctuations of circulating hormones, but also introduce an external stressor, due to the injection itself, and may artificially elevate inflammation at the injection site (4, 6). Slowly dissolving hormone pellets can be implanted subcutaneously (22). These release a constant

amount of hormone into circulation and resolve concerns due to injection. A major limitation of this method is that the supplied hormone concentration is a product of the exposed surface area, which decreases in size as the pellets dissolve. This results in an ever decreasing dosage of drug delivery over time. Silastic capsules filled with the hormone of interest provide the same benefit as pellets, with the additional benefit of releasing a constant hormone concentration until the capsule is empty. This is due to the hydrophobic diffusion of hormone across the capsule membrane at a constant rate. It has been previously determined that the equivalent of 20 mm Silastic capsules deliver a daily average testosterone concentration as what is normal in an adult male rat (9). For purposes of this study, gonadectomy with Silastic capsule hormone replacement was selected to maintain a constant hormone concentration without increasing the risk of inducing a daily inflammatory response by injection.

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CHAPTER II

ANDROGENS MODULATE CHRONIC INTERMITTENT HYPOXIA EFFECTS ON BRAIN AND BEHAVIOR

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** to be submitted to Journal of Clinical Investigation*

Abstract

Sleep apnea is associated with decreased testosterone and increased risk of comorbidities, such as Alzheimer's disease (AD) and Parkinson's disease (PD). A rodent model of the hypoxic events of sleep apnea, chronic intermittent hypoxia (CIH), elevates oxidative stress in male rats, while decreasing testosterone, which may underlie sleep apnea associated AD/PD comorbidities. Key features of AD/PD include low testosterone, elevated oxidative stress, and inflammation. Therefore, reduction of testosterone by CIH may increase neuronal dysfunction. Male rats were assigned to one of four hormone groups: 1) gonadally intact, 2) gonadectomized, 3) testosterone supplemented, or 4) dihydrotestosterone (DHT) supplemented. Memory and motor task assessment followed one week of CIH. Brain regions associated with AD/PD (substantia nigra, entorhinal cortex, hippocampus) were examined for oxidative stress and inflammatory markers. Gonadally intact rats exhibited elevated oxidative stress due to CIH, but no significant memory and motor impairments. Gonadectomy increased memory impairments. Testosterone replacement prevented all CIH-induced changes. In contrast, DHT was not protective, as evidenced by exacerbated oxidative stress under CIH. Further, these effects resulted in significant spatial memory impairment. These results indicate androgens can have both neuroprotective and detrimental effects under CIH. This may have clinical relevance for men with untreated sleep apnea.

Introduction

Sleep apnea (SA) is estimated to affect about a quarter of the United States population, and is often undiagnosed (43, 66, 80). The physiological causes of SA can be central or mechanical in nature (21). One measure of the severity of SA is the apnea/hypopnea index (AHI), which quantifies the hourly rate an apnea or a hypopnea occurs during sleep. The severity of SA is often defined as mild for AHI = 5 – 15, moderate for AHI = 15 – 30, and severe for AHI > 30 (75). In addition to hypoxic events and disrupted sleep patterns, patients with SA often experience a dysregulation in inflammation and oxidative stress (8, 11, 33, 50, 51, 56).

Several comorbidities are associated with SA. People diagnosed with SA are at higher risk to develop hypertension, metabolic disorders, and neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (34, 43, 51, 77, 81, 84, 96). Inflammation, oxidative stress, and low testosterone are key characteristics of all these disorders (20). SA increases oxidative stress and inflammation at early stages, and it is possible this initial rise in oxidative stress and inflammation may be contributing factors to later disease outcomes.

Sex differences are observed in SA. Regardless of ethnicity or race, men are more likely to be diagnosed with SA (69, 70, 72, 78, 87). Interestingly, aging is correlated with both incidence and severity of SA in men (5). While an increase in SA incidence is observed in post-menopausal women, the same trend is not observed for severity (5, 66, 97). Men with SA commonly report symptoms of low testosterone, which can be reversed by treatment of SA (99). These observations indicate androgens and estrogens may be contributing mechanisms underlying SA pathology (87).

The rodent model of chronic intermittent hypoxia (CIH) is used to study the hypoxic events experienced by patients with SA (31). Similar to what is experienced by men with SA,

male rats undergoing CIH exhibit sustained hypertension in addition to elevation of oxidative stress and inflammation (32, 45, 61, 82, 83). Additionally, gonadally intact female rats do not become hypertensive in response to CIH, which is consistent with the clinical phenotype of women with sleep apnea (38). These findings underscore the hypothesis that sex hormones underlie SA oxidative stress and inflammation. In addition to systemic effects, CIH can influence the CNS of male rats (74). Neuronal activation has been documented in hypothalamic and brainstem nuclei responsible for homeostatic behaviors (19, 45). Recently, we reported oxidative stress occurs within the substantia nigra (SN), the entorhinal cortex (ETC), and the hippocampus of male rats after one week of CIH exposure at an AHI of 10 (83). Damage to these brain regions has been implicated in pre-clinical stages of different neurodegenerative diseases (13, 14, 52, 73). It is not yet known if the observed increase in oxidative stress and inflammation in these regions is contributing to neuronal dysfunction leading to memory or fine motor deficits, or to what extent the influence of sex hormones may play in these hypoxic events.

While estrogens have been well established as protective against cardiovascular (23, 38) and neurodegenerative disorders (2, 30), the role of androgens is less defined (2, 4, 35, 39, 49). *In vitro* studies suggest testosterone can be either protective or damaging to neurons, depending on the concentration of oxidative stress present at the time of hormone administration (39, 40). Testosterone can be metabolized into two hormones: estradiol, via the enzyme aromatase, and dihydrotestosterone (DHT), via 5α-reductase (65, 86). Each of these metabolites activates different signaling cascades through its effect on its cognate receptor, resulting in testosterone initiating a broad range of cellular effects (67, 76, 89).

Testosterone is chiefly known for promoting male secondary sex characteristics (46). More recently, physiological testosterone has been linked with quality of life indices, such as

maintaining memory (42, 101). While men do not experience a drastic decline of their primary sex hormone, testosterone, as women do with estrogen, they do experience a slow decline in androgens as they age (1). That loss has been associated with a rise in cardiovascular, metabolic, and neurodegenerative disorders (68), prompting a recent surge in testosterone replacement therapy prescriptions (68). It is possible the low testosterone some men experience with SA represents a loss of protection, and it could be a major contributor to the increased risk of comorbid disorders.

The purpose of this study is to determine how testosterone contributes to early neurodegenerative biomarker and behavioral outcomes induced by CIH in male rats. We hypothesized 12-day exposure to CIH would be sufficient to induce behavioral deficits in memory and motor tasks. Additionally, we hypothesized these effects would be exacerbated by androgen supplementation. To investigate this, male rats were separated into various androgen groups by gonadectomy and exogenous hormone administration followed by exposure to CIH or normal room air conditions (figure 7). Following seven days of CIH, all groups underwent behavior testing to assess memory and motor skills, while continuing CIH treatment. We report CIH increased oxidative stress and impaired spatial memory recall, while exogenous testosterone administration blocked early CIH-induced damage. Interestingly, CIH damage was exacerbated in animals given the non-aromatizable androgen metabolite, DHT. In fact, the observed behavioral deficits due to CIH were most prominent in the rats exposed to DHT.

Results

CIH increases plasma oxidative stress

Rats were assigned to one of four hormone groups: gonadally intact (INTACT), gonadectomized (GDX), gonadectomized with testosterone supplementation (TRT) or gonadectomized with dihydrotestosterone supplementation (DHT). Rats from each hormone group were then randomly assigned to be exposed to either normal room air (NORM) or CIH during sleep for 12 days (figure 7). AOPP was significantly, but independently affected by both hormonal status ($F_{3,90} = 3.568$, $p < 0.05$) and CIH exposure ($F_{1,90} = 6.304$, $p < 0.05$). Although an AHI = 8 was used in this study, as opposed to AHI = 10 in our previous study to avoid possible ceiling effects, we observed similar findings on oxidative stress (83). INTACT-CIH rats experienced a significant 20% increase of oxidative stress in comparison to NORM rats (figure 8). Oxidative stress was further exacerbated in DHT-CIH rats, resulting in a 40% increase. TRT blocked CIH induced oxidative stress. No significant effects of CIH on GDX were observed.

CIH alters behavioral outcomes in rats given DHT

The Morris Water Maze (MWM) was used to explore the cognitive behavioral effects of CIH and hormone administration mediated by the hippocampus. Rats with damage to hippocampal neurons exhibit impairments in spatial memory retention (91). Target location latency was significantly affected by both CIH and hormonal status ($F_{3,60} = 4.711$, $p < 0.05$) (figure 9a). Under normoxic conditions, GDX rats took significantly longer to reach the target area than any other group, and these results were not affected by CIH. CIH significantly increased the latency to locate the target only in the DHT group.

CIH did not affect the swimming distance by any treatment group ($F_{1,57} = 0.215$, $p = 0.64$) (figure 9b). This indicates the observed differences in probe latency were not due to deficits in swimming ability among the groups. Additionally, there were no significant differences due to CIH in mean latency during the last training session ($F_{1,60} = 0.202$, $p = 0.66$). Therefore, CIH induced spatial memory retention deficits only in DHT rats, while GDX rats had overall impaired memory retention.

The lateral ETC is involved in development of short-term contextual memory, and has been documented to be damaged during pre-clinical phases of AD (22, 44, 93). Damage to the lateral ETC can be assessed in the novel object task. In this task, rats with an intact ETC spend more time with a novel object than with an object they were previously exposed to. Conversely, rats with damage to the ETC spend an equal or less amount of time with the novel object (93). This indicates ETC-compromised rats do not retain detailed, short-term, contextual memory, as compared to rats with an intact ETC. In this experiment, the novel object task was significantly influenced by the rat's hormonal status ($F_{3,75} = 4.601$, $p < 0.05$), but not CIH ($F_{1,75} = 0.965$, $p = 0.329$) was observed in the novel object task (figure 10a). Under normoxic and CIH conditions, DHT rats spent significantly more time with the novel object than the original object, in comparison with all other hormone groups. There were no significant differences in distance traveled during the assay between any treatment group (figure 10b), demonstrating neither hormone status ($F_{3,91} = 1.185$, $p = 0.32$) nor CIH ($F_{1,91} = 1.096$, $p = 0.298$) caused gross motor impairments.

A significant loss of cells in the SN results in movement disorders (14). To investigate if CIH-induced oxidative stress and inflammation in the SN altered fine motor impairment, a modified version of the open field assay was used. The addition of an elevated mesh platform

required use of fine motor skills during exploration of the open field. The number of falls was significantly affected by hormonal status ($F_{3,83} = 4.757$, $p < 0.05$). Specifically, DHT rats experienced more falls than any other group, regardless of CIH exposure (figure 10c). No differences due to CIH or hormone were observed in total rearing (CIH: $F_{1,92} = 0.848$, $p = 0.36$; hormone: $F_{3,92} = 1.518$, $p = 0.215$) or independent rearing (CIH: $F_{1,92} = 2.057$, $P = 0.16$; hormone: $F_{3,92} = 0.964$, $p = 0.413$).

CIH differentially alters protein expression in a regionally-specific manner

Because alterations to behavior were only observed in the INTACT, GDX, and DHT groups, brain tissue from these hormone groups were used for protein analysis. Oxidative stress in the SN, ETC, and hippocampus has been implicated in the early stages of neurodegeneration (55, 58). Western blot analysis was used to probe for protein levels of two indicators of oxidative stress, spectrin cleavage and NADPH oxidase (NOX1) expression. The scaffolding protein, spectrin, can be cleaved into a 145 kDa fragment by calpain, under oxidative stress, or a 120 kDa fragment by caspase 3, which is indicative of apoptosis (95). NOX1 generates ROS, which can contribute to oxidative stress and has been shown to be up-regulated in hypoxic conditions (61, 62, 92).

Significant differences in the calpain-cleaved 145 kDa fragment of spectrin were observed in all three brain regions (figure 11). Within the SN, calpain cleavage of spectrin was significantly affected by CIH and hormonal status ($F_{2,19} = 3.291$, $p < 0.05$) with main effects of both CIH ($F_{1,19} = 5.688$, $p < 0.05$) and hormone status ($F_{2,19} = 22.532$, $p < 0.05$) (figure 11a & 11b). DHT rats had higher calpain activity than all other hormone groups, regardless of CIH exposure. CIH significantly increased calpain activity in INTACT rats compared to INTACT-NORM rats. 120 kDa caspase-cleaved bands were not detectable in the SN. This suggests CIH

increases oxidative stress in the SN of INTACT rats, but cell death markers are not observable in this region at this time point.

In both the ETC ($F_{2,17} = 17.976$, $p < 0.05$) and hippocampus ($F_{2,19} = 48.791$, $p < 0.05$), calpain cleavage of spectrin was significantly affected by hormonal status, in which DHT rats had significantly more calpain cleavage of spectrin, as indicated by the 145 kDa band (figure 11c & 11e). Unlike what is observed in the SN, CIH did not have an effect on calpain activity in the ETC or hippocampus (figure 11a, 11c, & 11e). This indicates that oxidative processes in brain tissue are controlled in a regionally specific manner.

Caspase-3 cleaved 120 kDa fragments are detectable in both the ETC and hippocampus, unlike what is observed in the SN. In the hippocampus, caspase-3 cleavage activity was significantly affected by hormonal status ($F_{2,18} = 10.886$, $p < 0.05$), but not in the ETC ($F_{2,27} = 1.905$, $p = 0.168$) in which DHT caused a significant increase in the 120 kDa fragment of spectrin compared to both the INTACT and GDX groups (figure 11a & 11f).CIH did not cause significant differences in the 120 kDa fragment in either the hippocampus ($F_{1,18} = 0.011$, $p = 0.917$) or the ETC ($F_{1,27} = 0.002$, $p = 0.963$) of any hormone groups (figure 11a, 11d, & 11f). Similar to the SN, it appears caspase-3 is not activated in response to CIH exposure.

NOX1 expression was also affected by androgens in a regionally specific manner. In the SN, NOX1 expression was not altered in response to either CIH ($F_{1, 17} = 0.134$, $p = 0.719$) or hormone status ($F_{2, 17} = 1.797$, $p = 0.196$) (figure 12a & 12b). Similar to what was observed with spectrin cleavage, NOX1 expression was significantly affected by hormonal status in the ETC ($F_{2, 15} = 131.528$, $p < 0.05$) and hippocampus ($F_{2, 20} = 25.737$, $p < 0.05$) of male rats (figures 12d & 12e, 12g & 12i). In both regions, DHT rats had significantly higher NOX1 expression than INTACT rats or GDX rats (figure 12e & 12h).

Androgens modulate astrocyte presence in CNS regions

Since our current study utilized CIH (AHI = 8) for 12 days, we wanted to determine if macrophage invasion may underlie an inflammatory response. Thus we examined markers of activated macrophages and microglia (cd11b) and astrocytes (GFAP). We found no evidence of cd11b staining in any of the brain regions of any treatment group (data not shown). However, GFAP protein was detectable in all 3 brain regions, indicating the presence of astrocytes (figures 12c, 12f, 12i). GFAP protein expression was observed to be significantly altered by hormonal status in all brain regions. In the SN ($F_{2,20} = 5.286$, $p < 0.05$), GDX rats exhibited significantly more GFAP expression than INTACT rats (figure 12c). In the ETC ($F_{2,14} = 27.137$, $p < 0.05$), DHT rats had significantly more GFAP expression than either INTACT or GDX rats (figure 12f). In the hippocampus ($F_{2,18} = 12.772$, $p < 0.05$), GDX rats had significantly lower GFAP expression than INTACT or DHT rats did (figure 12i). GFAP expression was not altered by CIH in any brain region (SN: $F_{1,20} = 0.026$, $p = 0.873$; ETC: $F_{1,14} = 0.194$, $p = 0.666$; HIPP: $F_{1,18} = 2.409$, $p = 0.138$).

CIH induces inflammatory dysregulation in a region-specific manner

In our previous study, 7 days of CIH at AHI = 10 resulted in an elevation of M1 activating pro-inflammatory cytokines over M2 activating anti-inflammatory cytokines in the plasma, SN, and ETC in Sprague-Dawley. Contrary to those observations, significant effects due to 12 days CIH at AHI = 8 were not observed in the circulation or in the ETC of Long-Evans, possibly due to not meeting an oxidative stress threshold to initiate an inflammatory response. In contrast, an inflammatory response was observed in the SN, which may be due to the SN having a lower oxidative stress threshold than the other brain regions studied.

In plasma, pro-inflammatory M1 cytokine expression was found to be significantly altered by hormonal status ($F_{2,13} = 6.427$, $p < 0.05$), but not CIH ($F_{1,13} = 0.029$, $p = 0.867$). GDX rats exhibited significantly lower M1 cytokines than either the INTACT or DHT rats (table 6). Similarly, anti-inflammatory M2 cytokine expression was significantly affected by hormonal status ($F_{2,13} = 10.070$, $p < 0.05$), but not CIH ($F_{1,13} = 1.496$, $p = 0.243$), resulting in GDX rats exhibiting significantly less M2 cytokines than the other groups. This suggests basal inflammatory signaling in male rats is highly dependent on the presence of androgens.

Similar to what was observed with oxidative stress, regional CNS differences were observed in inflammation. In the SN, M1 activating cytokine expression was significantly affected by both CIH ($F_{1,18} = 7.280$, $p < 0.05$) and hormone ($F_{2,18} = 30.820$, $p < 0.05$) (table 6). DHT rats had significantly more M1 cytokine expression than either INTACT or GDX rats, and CIH exposure significantly increased M1 cytokines. M2 cytokine expression in the SN was also significantly affected by hormonal status ($F_{2,18} = 8.548$, $p < 0.05$) but not CIH ($F_{1,18} = 0.126$, $p = 0.727$). DHT rats had significantly less M2 expression, than INTACT and GDX rats. This suggests that in the SN, DHT caused a pro-inflammatory environment by increasing M1 cytokines and simultaneously decreasing M2 cytokines. CIH appears to exacerbate the pro-inflammatory effect of DHT by further increasing M1 cytokines.

In the ETC, both M1 and M2 activating cytokines were significantly affected by hormonal status (M1: $F_{2,14} = 14.048$, $p < 0.05$; M2: $F_{2,14} = 17.854$, $p < 0.05$) (table 6). This resulted in both GDX and DHT rats expressing significantly less M1 activating cytokines than INTACT rats. Additionally, GDX rats had less M2 cytokines than INTACT rats, and DHT rats had significantly less M2 cytokines than either of the other two groups. This suggests both

estrogenic and androgenic signaling pathways are necessary for basal inflammatory signaling in the ETC.

In the hippocampus, M1 and M2 activating cytokines were significantly suppressed by CIH (M1: $F_{1,14} = 5.959$, $p < 0.05$; M2: $F_{1,14} = 5.985$, $p < 0.05$) (table 6). This suppression was the greatest in INTACT rats. Unlike the other brain regions, no effect of hormone was observed in M1 cytokines ($F_{1,14} = 1.280$, $p < 0.309$) or M2 cytokines ($F_{2,14} = 1.785$, $p = 0.204$) in the hippocampus. Under normoxic conditions, there was a non-significant trend of hormone for both M1 ($F_{2,8} = 4.487$, $p = 0.064$) and M2 ($F_{2,8} = 4.080$, $p = 0.076$) cytokines to be lower in the hippocampus of DHT rats.

Discussion

We hypothesized the oxidative stress and neuronal effects of CIH were influenced by androgens. This study used a slightly lower hypoxic exposure (AHI = 8) than our previous study (AHI = 10) to avoid a possible ceiling effect due to superimposing androgens. However, our results indicate CIH caused a similar increase in circulating oxidative stress (figure 8), as well as regional differences in the brain (figure 11 & 12) in agreement with our prior observations. To our knowledge, this is the first study investigating the effects of CIH on the Long-Evans rat strain. The main findings of this study include: 1) TRT blocked oxidative stress, but the non-aromatizable androgen, DHT, exacerbated it, 2) CIH induced spatial memory impairment in DHT rats, and 3) DHT increased susceptibility to oxidative stress and M1 pro-inflammatory cytokines in the CNS (table 6, figures 8, 9, 11 & 12). This suggests androgen signaling, in the absence of aromatized estrogen signaling, may render male rats susceptible to the effects of CIH.

Our data show that both CIH and androgens can increase circulating and brain region specific oxidative stress. CIH increased circulating oxidative stress (as measured by AOPP) in male rats, but not in hormone-deficient rats. Since testosterone can be metabolized into estradiol and DHT, we wanted to determine if the androgen pathway may be involved in this oxidative stress response to CIH. Indeed, CIH significantly increased oxidative stress in DHT rats, indicating androgens are involved. To further examine what impact CIH and androgens had on the brain, we investigated the response of oxidative stress-related proteins (NOX1 and calpain-cleavage of spectrin) in the ETC, hippocampus, and the SN. DHT increased oxidative stress markers in all brain regions examined. Surprisingly, we found a lack of response of NOX1 to CIH in all the brain regions examined. It is possible that the lower AHI used in our study may underlie this difference from other studies that have reported a role of NOX1 in CIH-induced

oxidative stress generation (62, 85). However, it is more probable a ceiling effect was reached, especially in the DHT group in which CIH was not able to further elevate NOX1 expression in the SN. This possibility is consistent with our data using the oxidative stress marker, calpain-cleavage of spectrin, which was elevated in the SN of gonadally intact male rats but did not further increase protein expression in the DHT group. The significant increase in calpain-cleavage observed in the SN, but not the other regions examined, may partially be due to the abundance of catecholaminergic neurons present within the SN, which are sensitive to oxidative stress (54). The neuronal composition of the SN is relatively homogeneous, in which 95% of the cells are dopaminergic neurons (48). In contrast, the ETC and hippocampus are composed of a wider variety of cell types, which may partially compensate for the oxidative effects of CIH in those regions (25, 41).

Both hormones and CIH contribute to neuronal oxidative stress pathways. Androgens can cause an increase in oxidative stress, as well as pro-inflammatory M1 cytokines. M1 signaling can, in turn, further increase oxidative stress (59). An accumulation of oxidative stress within neurons can be damaging and trigger apoptotic pathways (90). Oxidative stress has been implicated in pro-inflammatory responses to CIH (50, 79). Unpublished data from our lab using a neuronal cell line did not find a neuronal inflammatory response to oxidative stress. However, an inflammatory response to CIH was evident in the SN of INTACT, GDX, and DHT rats, and the hippocampus of INTACT rats, indicating that a non-neuronal cell type is mediating the inflammatory response. We did not observe evidence of either microglia or macrophages, but there were regional changes in astrocytes (figure 12). These results indicate the inflammatory response observed may be mediated by astrocytes. Regional astrocytes may serve to tune

neuronal responses within each system and this tuning may then underlie SA comorbidities, such as neurodegenerative diseases (Alzheimer's and Parkinson's diseases) and hypertension (51, 63).

Behavioral deficits associated with Parkinson's disease are not observed until 70-80% cell loss within the SN (14, 18), which is consistent with our behavioral data. We did not see any overt motor impairments (figure 10). However, we did observe fine motor impairments in the DHT group. The DHT group consistently experienced more falls in the modified open field than any other group (figure 10). Although oxidative stress and M1 signals were elevated by CIH in the SN, apoptosis induced cell death was not evident in the SN at this time point (figure 11). Our study indicates DHT or a more androgenic profile would increase motor function susceptibility to future impairments, such as long term CIH (sleep apnea) exposure.

The behavior most impacted by CIH in this study was memory. Unlike Parkinson's disease that requires cell loss before behavioral impairment, patients with Alzheimer's disease begin to exhibit clinical symptoms when a substantial number of their hippocampal cells have been impaired (12). Patients with sleep apnea are at a higher risk to develop cognitive disorders, such as Alzheimer's disease and vascular dementia (15, 28). In this study, DHT elevated oxidative stress in both the ETC and hippocampus, as well as increased apoptosis in the hippocampus. This indicates androgens can increase the sensitivity of neurons to a subsequent oxidative stressor, such as CIH. Indeed, CIH induced significant cognitive impairments in DHT rats, and thus androgens may underlie the SA-associated comorbidity of Alzheimer's disease. The lack of effects of CIH on GDX rats' behaviors in the novel object task could be due to the effects of androgens on novelty (60), resulting in this behavior test being inappropriate for assaying memory in androgenic rats. Overall, these findings are consistent with literature

demonstrating that both testosterone and estrogen are protective in the CNS and facilitate memory, while DHT can be damaging (2, 7, 9, 27, 65).

CIH can induce hypertension (82). Hypertension, itself, increases oxidative stress and inflammation, which can be further exacerbated by androgens (88, 90). (50). Interestingly, hypertension can increase the risk of developing cognitive disorders, such as vascular dementia (6, 57). The impact hypertension has on motor dysfunction is less defined and requires further investigation. The role of sleep apnea-induced hypertension on neurodegeneration is understudied.

Our findings are of clinical relevance to men with sleep apnea. Although exogenous testosterone (presumably via estrogenic signaling) in this study prevented oxidative stress and memory deficits, androgenic signaling by DHT exacerbated these measures. Men have higher androgen concentrations and are more likely to be diagnosed with sleep apnea than women (5, 21). These effects are unlikely to be exclusively due to a genetic influence based on prior studies showing women with polycystic ovarian syndrome (characterized by elevated testosterone) are more likely to have sleep apnea, hypertension, and cognitive disorders, suggesting elevated androgenic signaling contributes to risk (37, 71). This upregulation of oxidative stress, inflammation, and hypertension can create a vicious cycle that may underlie the comorbidities of sleep apnea. Of concern, is that these effects of CIH were observed after only one week, which is when CIH induced hypertension has been reported (45).

Many people with sleep apnea are undiagnosed (43, 53). Among the population diagnosed for sleep apnea, the recommendation for treatment of mild sleep apnea is optional, and large portions of the clinical population remain untreated or elect to halt compliance (3, 24).

These early processes could have serious consequences on brain and behavior, and may be a vital point for therapeutic intervention to prevent early neurodegenerative processes.

Methods

Animals: 100 adult Long-Evans male rats (250-275 g body weight, 50-57 d, Charles River) were individually housed in a temperature controlled environment until one-week post-surgery to allow for recovery from all surgical procedures. After recovery, they were pair-housed for the duration of the study. Lights were set on a 12:12 h reverse cycle with lights off at 0900 h. Food and water were provided *ad libitum*. To acclimatize rats to operator handling and reduce stress responses during behavior testing, all rats were handled 3 times per day beginning the morning of the eighth day after arrival. This continued 5 days a week. All experiments were approved by the IACUC at UNT Health Science Center and conducted according to NIH guidelines on laboratory animals.

Surgical procedures: To investigate the contributions of testosterone on the effects of CIH, rats were randomly assigned to one of 4 hormonal groups: gonadally intact control with a cholesterol-filled Silastic capsule implant (INTACT), gonadectomized control with a cholesterol-filled Silastic capsule implant (GDX), gonadectomized with two testosterone-filled Silastic capsule implants (TRT), or gonadectomized with one dihydrotestosterone-filled Silastic capsule implant (DHT). For all surgery procedures, rats were lightly anesthetized with isoflurane (2-3%). All rats underwent either gonadectomy or sham surgery. Hormone replacement was achieved with subcutaneous Silastic capsule implants (1.47 mm i.d. x 1.96 mm o.d. x 10 mm length, Dow Corning, Midland, MI) filled with either crystalline testosterone, dihydrotestosterone, or cholesterol (Steraloids, Newport, R.I.) as previously described (94). Two testosterone capsules were used to maintain physiological levels of testosterone in male rats (64, 94). Based on previous studies, we used one Silastic capsule containing DHT (26) to investigate the contribution of the non-aromatizable androgen under oxidative stress conditions.

Chronic intermittent hypoxia protocol: Following pair-housing, rats from each hormone group were randomly assigned to receive either CIH or NORM exposure during sleep, resulting in 8 treatment groups: INTACT-NORM (n = 26), INTACT-CIH (n = 26), GDX-NORM (n = 10), GDX-CIH (n = 8), TRT-NORM (n = 6), TRT-CIH (n = 8), DHT-NORM (n = 8), & DHT-CIH (n = 8). Home cages were placed into Oxycycler chambers (76.2 x 50.8 x 50.8 cm, BioSpherix, Lacona, NY, USA) and rats were allowed to acclimatize to the Oxycycler under NORM conditions for one week. Oxygen was reduced from 21% to 10%, then returned to 21% in 8 minute cycles from 2100 – 0500 for 12 days, as previously described (94) (figure 7).

Morris Water Maze: Beginning at 0900, on the eighth day of CIH, all rats were trained to swim to a visible platform in a pool filled with room temperature water and to remain on the platform for 20 s, until removed by the operator. Morris Water Maze training began the following morning at 0900. Training consisted of 3 trials per day for 3 days with a 10-minute inter-trial interval per rat. For each trial, a rat was placed into the pool filled with opaque water at a randomly assigned point. Each point was equidistant from an underwater platform (target), which was 1 cm below the surface of the water. Rats were then allowed 90 s to locate the target. The trial ended when either the rat located the target and climbed onto it, or 90 s had passed, whichever occurred first. Time and distance to locate the target was recorded using ANY-maze software (v. 5.14, Stoelting Co.). Rats which did not locate the target during a particular trial were guided to the target by means of the operator tapping on the target until the rat swam to it. Once the target was located, each rat was allowed to sit on the platform and observe visual cues placed on the walls to aid in formation of spatial memory for 20 s. After the 20 s passed, the rat was removed from the water maze and placed into a carrier to dry and await the next trial. The target remained in the same location throughout all 3 days of training.

At 0900 on the 12th day of CIH, each rat was administered a probe trial to test for spatial memory retention. During the probe trial, the underwater platform was removed. Each rat was placed into the water at one of the pre-determined random entry points and allowed 30 s to swim to the target location and search for the platform. At the end of 30 s, the platform was returned to its original location and the rat once again had 20 s to sit on the platform to reduce stress from failure to locate the platform in subsequent behavior testing. Rats were then returned to their home cages for 1 h to rest prior to further behavioral testing. Time and distance traveled to the target location during the 30 s, was recorded and used for statistical analysis as indicators of spatial memory retention and gross motor function.

(modified) Open field assay: An open field apparatus (16" (W) x 16" (D) x 15" (H), San Diego Instruments) was modified with a wire mesh platform raised 2 cm above the floor to assay fine motor skill impairment. Laser beams were placed both above and below the plane of the platform to record horizontal movement and falls through the mesh platform. Movements were recorded by PAS software (PAS v. 1.0.0.0, San Diego Instruments). The operator manually recorded the number of nose-pokes through the wire mesh platform and vertical exploratory events (rearing), both assisted (defined by placement of a forepaw on a maze wall or a hind paw on the solid floor of the maze) and unassisted. The number of nose-pokes was subtracted from the fall count to provide an accurate count of falls for statistical analysis. Each rat was placed into the center of the open field on the mesh platform and allowed 5 minutes to explore the space. Following testing, rats were returned to their home cages for at least 1 h prior to further assessments. The number of falls and the number of rearing events (assisted, unassisted, and total) were quantified and used for statistical analysis.

Novel object assay: An open field (24" (W) x 24" (D) x 12" (H)) was used with 2 objects placed in two adjacent corners (93). Activity in the open field was monitored and recorded on ANY-maze software (v. 5.14, Stoelting Co.). This test consisted of 2 trials, one h apart. In the first trial, both objects were similar in size and nature. The rat was placed into the open field facing the wall opposite the two objects to avoid object bias and allowed to explore the space and the objects for 5 minutes. At the end of 5 minutes, the rat was removed from the open field and returned to a carrier to rest for 1 h. Prior to the second trial, one object was replaced with a novel object, different in both size and shape from the first two objects. The rat was then placed back into the field in the same location as before and allowed 3 minutes to explore the field. At the end of 3 minutes, the rat was returned to the carrier for transport back to its home cages at the end of testing. The distance traveled, time spent with each object, latency to approach each object, and number of approaches were recorded and used for statistical analysis.

Sample collection: Between 0900 and 1100 of the thirteenth day, rats were deeply anesthetized with isoflurane, then euthanized by decapitation. Trunk blood was collected in chilled EDTA coated tubes, then allowed to sit on ice and centrifuged to collect plasma for biochemical analysis as previously described (83). Additionally, brains were rapidly harvested, placed on ice, and immediately sliced into 1 mm coronal sections using a commercially available matrix (Ted Pella, Inc., Redding, CA). To ensure specificity of the different brain regions, the SN was collected by micro-punches using blunt 20 gauge needles attached to 1 ml syringes, and the ETC and hippocampus were dissected. All tissue samples were placed in 1.7 ml microcentrifuge tubes and then flash frozen on dry ice. All samples were stored at -80° C for later analysis.

Tissue Homogenization: Each tissue sample was homogenized according to previously published methods (83). Protein concentrations were determined by Pierce BCA assay according

to manufacturer's protocol (ThermoFisher Scientific). Lysate was aliquoted and stored in microcentrifuge tubes at -80 for further analysis.

Advanced oxidative protein products (AOPP) assay: Circulating oxidative stress was assayed using the Advanced Oxidative Protein Products assay kit from Cell Biolabs, Inc. as previously described (83). Values are expressed as a percent of the mean INTACT-NORM control values for each run using the formula (sample uM concentration / mean of INTACT-NORM uM concentrations) * 100. Results are reported as mean ± SEM.

Western blot: Equal volumes of denatured tissue samples containing 20 ug protein were loaded into a BioRad 4-20% polyacrylamide gel. They underwent electrophoresis at 25 mA in a Tri-glycine transfer buffer followed by overnight transfer onto a PVDF membrane at 60mA. Following 30 minutes washing, membranes were blocked for 30 minutes with 5% nonfat milk in TBS-Tween (TBST) at room temperature. Membranes were then transferred to 1% nonfat milk TBST solutions containing specific primary antibodies (NOX1, Santa Cruz sc-25545 1:200; spectrin, EMD Millipore MAB1622 1:5000; GFAP, Sigma-AldrichG3893 1:2000; GAP-DH, GeneTex GT627408 1:10,000) and incubated overnight at 4°C. In contrast to other primary antibodies, solutions containing primary Ab for cd11b (Novus Biologicals NB110-89474 1:500) were prepared in 5% milk TBST solution and allowed to incubate at RT for 1 h according to manufacturer's protocol (Novus Biologicals). Afterwards, membranes were washed in 10 minute increments for 30 minutes, and then incubated in 1% mild TBST secondary antibody solutions (goat anti-rabbit 1:5000, goat anti-mouse 1:10,000 or 1:2000) at room temperature for 1 h. Protein bands were visualized using West Pico enhanced chemiluminescence detection assay (Thermoscientific) on an Syngene G:Box system using FlourChem HD2 AIC software as previously described (29). NIH Image J software (version 1.50i) was used to quantify band

densitometry and values from AR, NOX1, and GFAP were normalized to GAP-DH values using the equation (mean gray value for protein / mean gray value of GAP-DH) *100. For spectrin cleavage analysis, bands representing cleavage by either calpain (145 kDa) or caspase-3 (120 kDa) were normalized to total spectrin (260 kDa) values by the equation (mean gray value of specific kDa band / mean gray value of 260 kDa band) * 100.

Cytokine multiplexing: Plasma, cell lysate (90ug), and brain tissue homogenate (120ug) samples were used to quantify secreted cytokines using the Bioplex Rat Th1/Th2 12-Plex kit available from Biorad and fluorescence was measured on a Luminex platform (MAGPIX, Luminex Corporation, Austin, TX) (table 6) (83). Activated astrocytes have been observed to increase secretion of IL-1β, IL-6, TNF-α, and GM-CSF under inflammatory conditions (10, 16, 100). These same proteins are documented to recruit and activate pro-inflammatory macrophages (M1) (17, 36, 47, 98), while IL-10, IL-13, and IL-4 cytokines are released to recruit and activate anti-inflammatory macrophages (M2). Homeostasis often depends upon maintaining the proper ratio of these inflammatory responses, while degenerative processes accompany the dysregulation of pro- and anti-inflammation. Therefore, cytokine concentrations from this assay were averaged into M1-activating (IL-1β, IL-6, TNF-α, and GM-CSF) and M2-activating (IL-10, IL-13, and IL-4) cytokine groups for statistical analysis of inflammatory dysregulation. Values are reported as mean M1 or M2 concentration ± SD (pg/ml).

Statistical analysis: IBM SPSS (SPSS v. 24, IBM, 2016) was used for statistical analysis. 2-way ANOVAs were run on all assays with CIH and hormone status as fixed factors. Fisher LSD post hoc analysis was performed to determine differences between treatment groups. Results are shown as mean ± SEM, unless otherwise stated. Statistical significance was at $p \leq 0.05$.

Acknowledgements

The authors would like to thank E. Nicole Wilson for her technical assistance in animal studies and immunohistochemistry.

The research reported here was supported by the National Institutes of Health (NIH) R01 NS0091359 to RLC and NIH training grant T32 AG 020494 to BDS.

FIGURE 7

EXPERIMENTAL TIMELINE

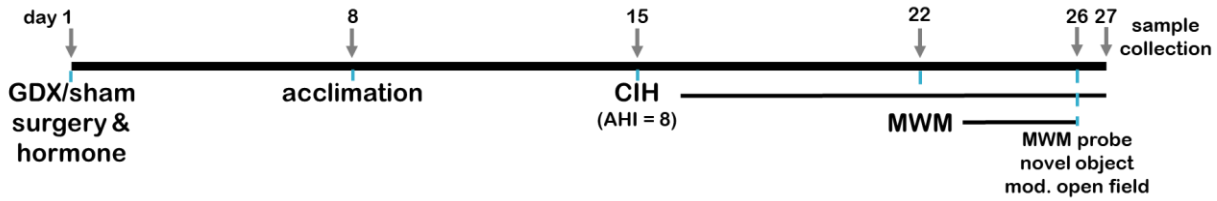

Figure 7: Following one week of acclimation to reverse lighting in the animal facility, male rats underwent surgery for assignment to hormonal groups. This was followed by one week of recovery, then one week of acclimation to pair-housing and the Oxycycler chambers, prior to the start of CIH. Behavior testing began at 0900 of the 8th day of CIH, during the rats' normal wake-phase. Testing continued for 5 days with CIH exposure continuing during the rats' sleep phase each night. Sample collection occurred at 0900 on the day after the final behavior test.

FIGURE 8

OXIDATIVE STRESS RESPONSE TO CIH AND ANDROGENS

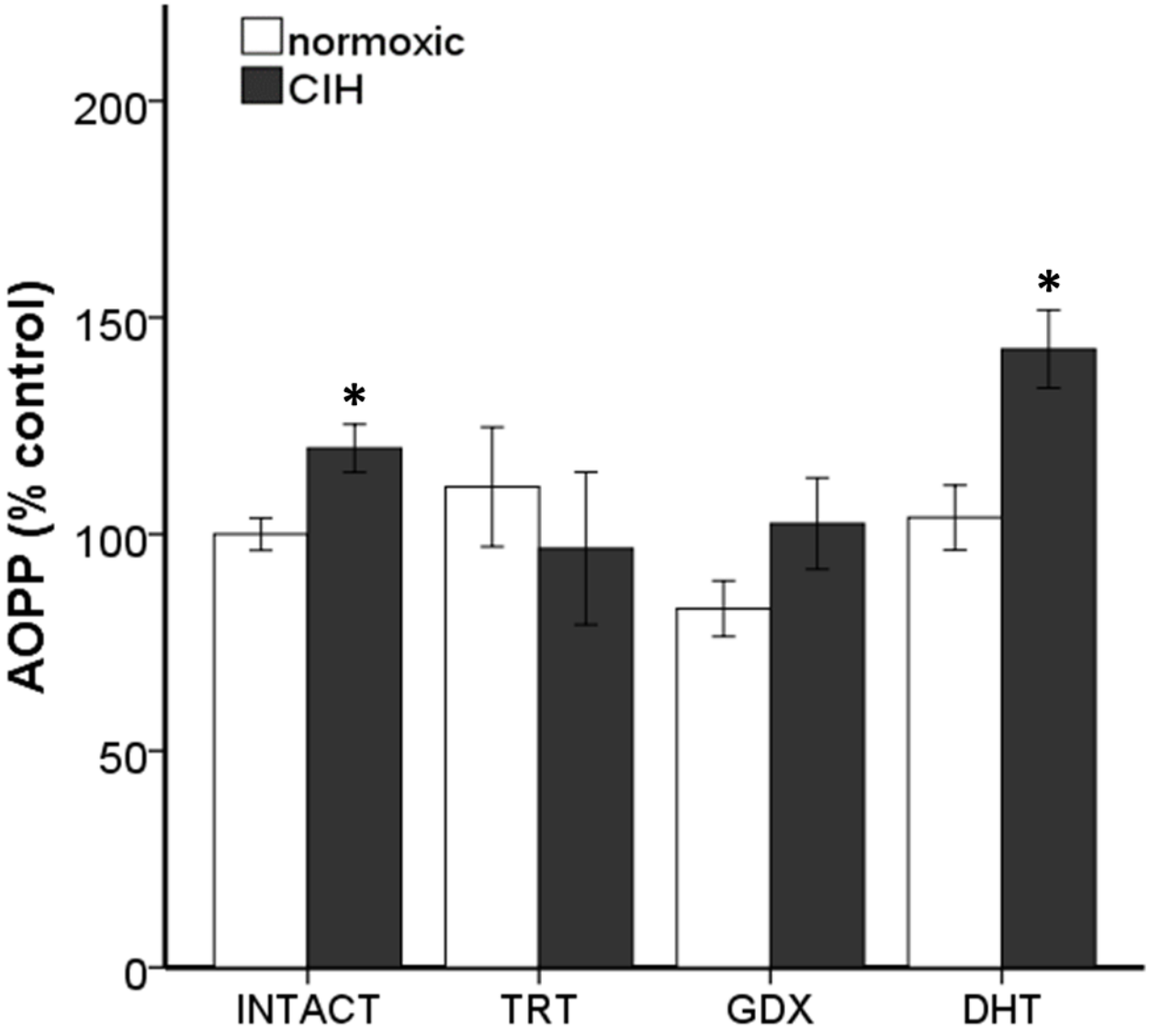

Figure 8: CIH cause an increase in circulating oxidative stress as measured by Advanced Oxidative Protein Products (AOPP) assay. Intact rats exposed to CIH experienced a significant increase in oxidative stress, which was not observed in rats administered exogenous testosterone (TRT) or gonadectomized (GDX) rats. Administration of dihydrotestosterone (DHT) exacerbated the CIH effect. *Results are shown as mean ± s.e.m. $p < 0.05$; * vs. normoxic*

Figure 9: Chronic intermittent hypoxia caused significant impairment in the probe trial of the Morris Water Maze. **A)** GDX male rats exhibited significant memory impairments which were not due to CIH exposure. DHT significantly exacerbated the memory impairment from CIH. B) The observed differences were not due to swimming ability. *Results are shown as mean ± s.e.m. $p < 0.05$; * vs. normoxic, # vs. all other groups*

FIGURE 9

CHRONIC INTERMITTENY HYPOXIA AND ANDROGENS AFFECT MEMORY RETENTION IN MALE LONG EVANS RATS

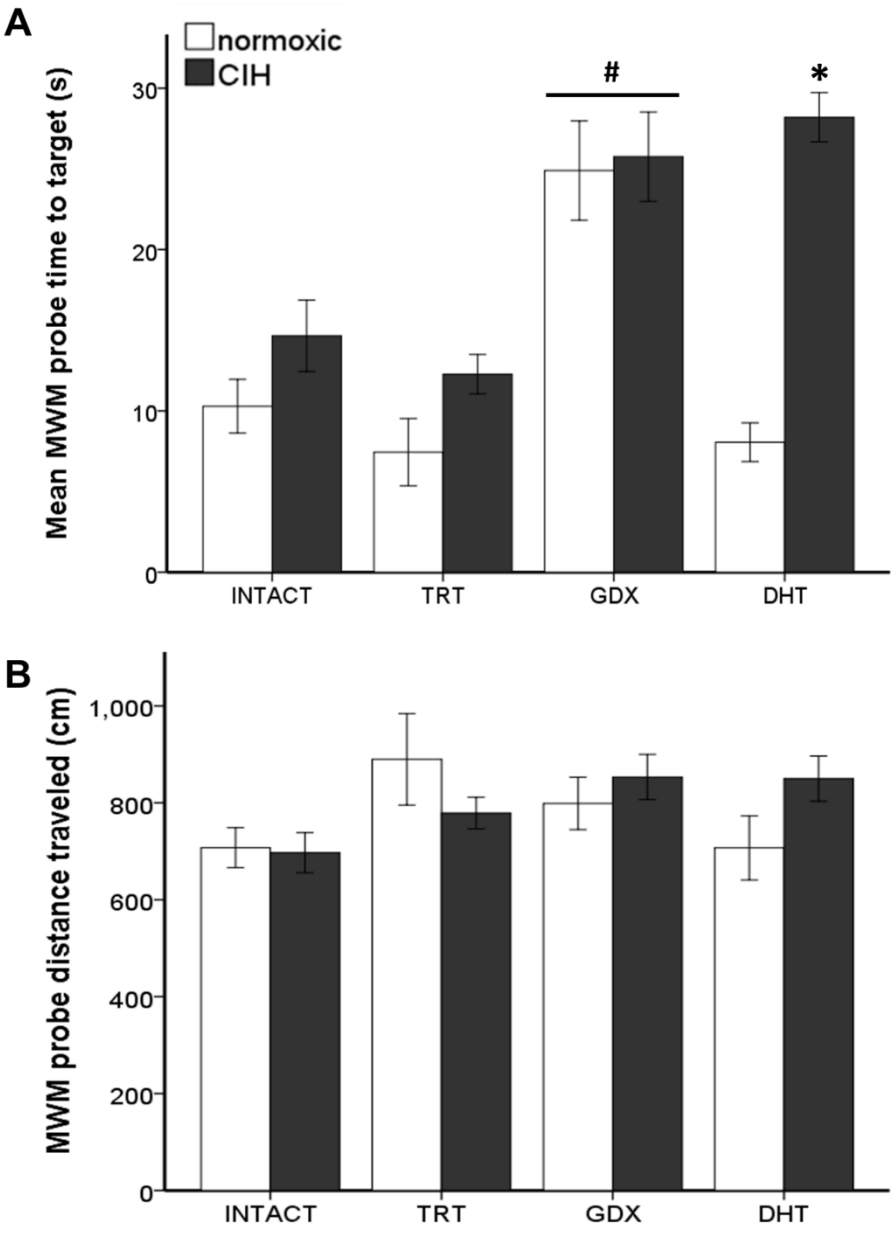

Figure 10: DHT causes alterations in results of the novel object and open field assays. **A)** CIH was not observed to impair lateral ETC function as measured by time spent with a novel object in the novel object task. Administration of DHT significantly improved time spent with the novel object. **B)** Gross motor ability was not impaired by CIH or hormone status as seen by the distance traveled during the novel object assay. **C)** DHT, but not CIH impaired fine motor ability of rats to balance on a wire mesh platform in the modified open field assay. *Results are shown as mean ± s.e.m. $p < 0.05$; # vs. all other groups*

FIGURE 10

DIHYDROTESTOSTERONE AFFECTS BEHAVIOR

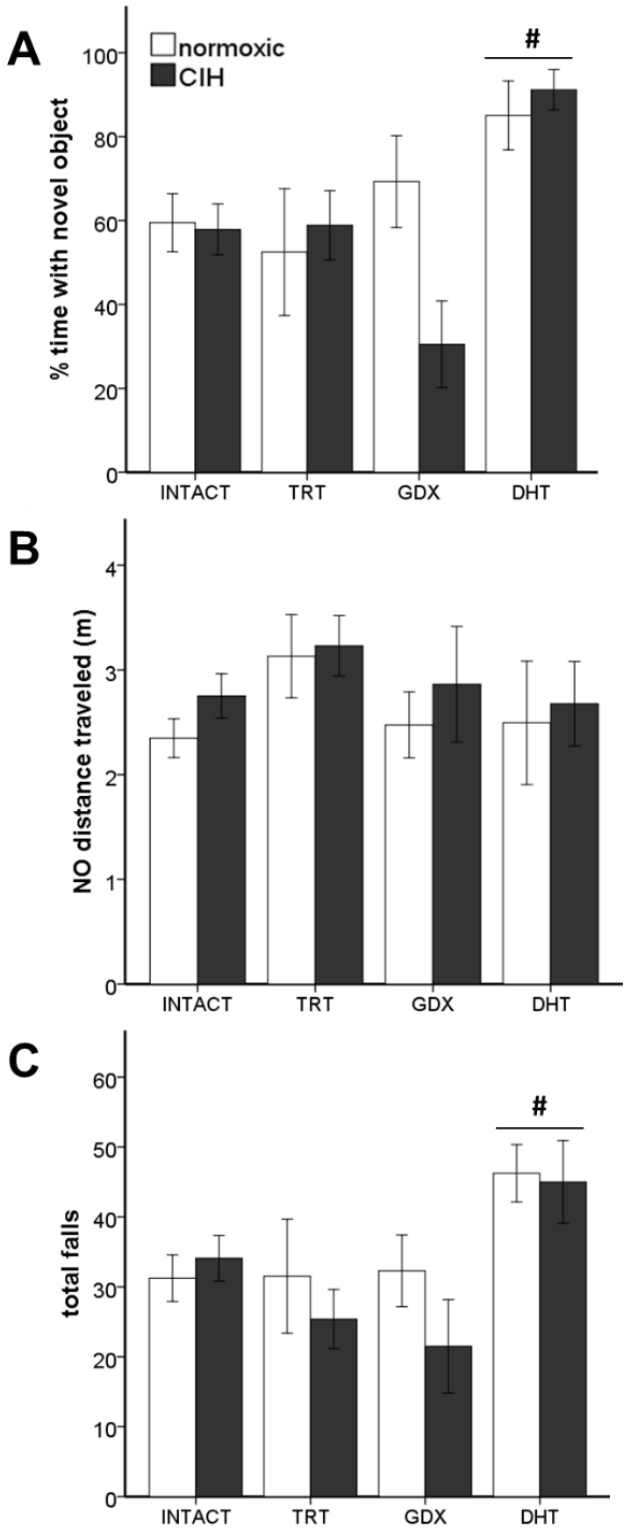

Figure 11: Calpain and caspase-3 cleavage of spectrin occurs differently within brain regions. **A)** Representative images of western blots of tissue from the substantia nigra (SN), entorhinal cortex (ETC), and hippocampus (HIPP) of intact, GDX, or DHT supplemented rats exposed to CIH. Full-length spectrin is as 260 kDa, calpain-cleaved fragments are at 145, and caspase-3-cleaved fragments are at 125 kDa. Densitometry for the fragments was normalized to the full length (cleaved fragment/full length). **B)** In the SN, a significant increase in calpain cleavage of spectrin due to CIH was observed in intact male rats. DHT administration significantly increased calpain cleavage, regardless of CIH exposure. No caspase-3 cleavage was evident in the SN. **C)** In the ETC, a significant decrease in calpain cleavage of spectrin due to CIH was observed in intact male rats. DHT administration significantly increased calpain cleavage in the ETC as well, regardless of CIH exposure. **D)** Although caspase-3 cleavage was evident in the ETC, no differences due to CIH or hormone were observed. **E)** In the hippocampus, a significant increase in calpain cleavage of spectrin due to CIH was observed in GDX male rats. DHT administration significantly increased calpain cleavage, regardless of CIH exposure. **F)** DHT also increased caspase-3 cleavage in the hippocampus. *Results are shown as mean ± s.e.m. $p < 0.05$; * vs. normoxic, # vs. all other groups*

FIGURE 11

CHRONIC INTRMITTENT HYPOXIA AND ANDROGENS CHANGE CALPAIN CLEAVAGE IN THE BRAIN

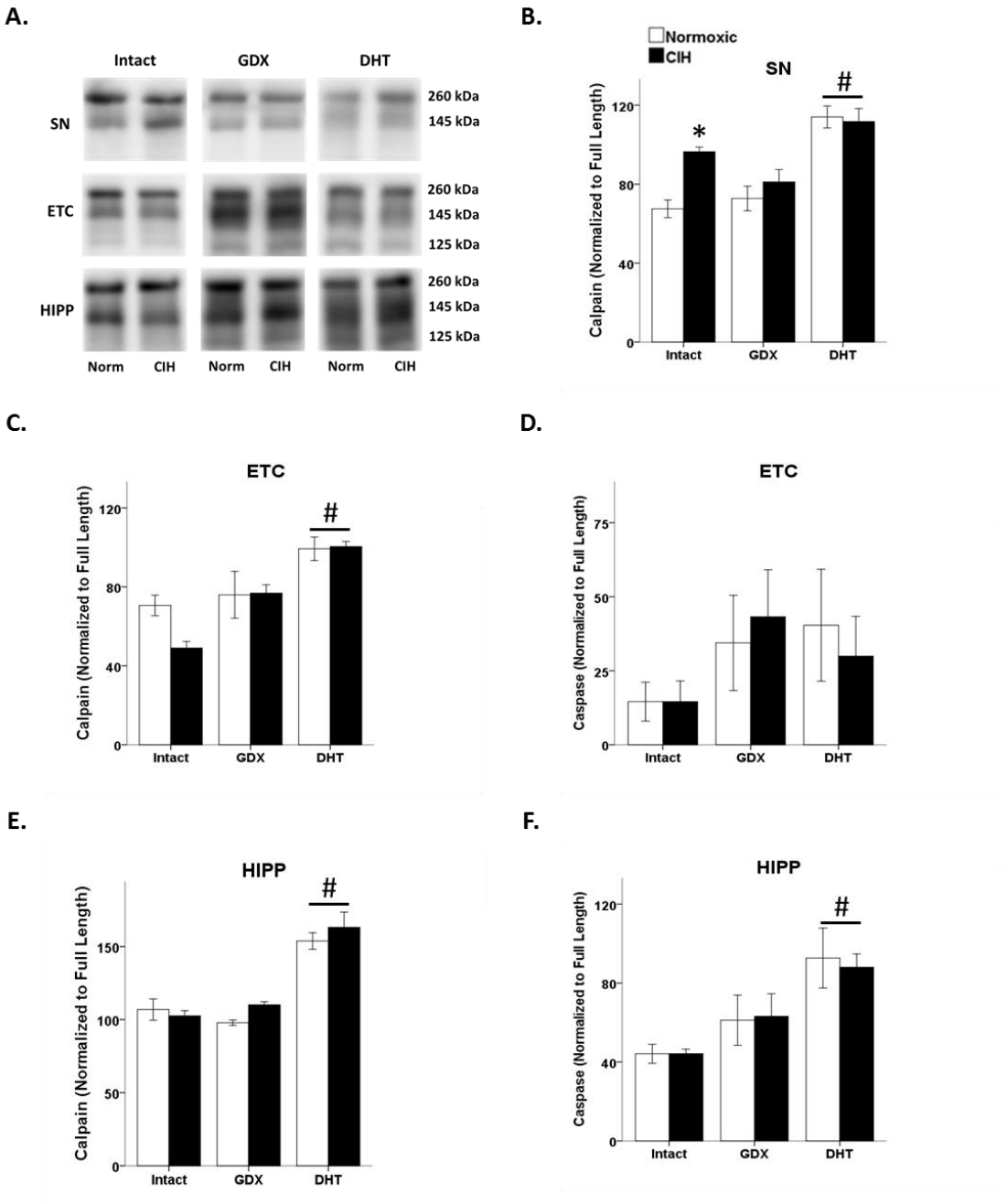

Figure 12: Hormone, but not CIH causes a change in oxidative stress and astrocytes in brain regions. **A)** Representative western blots results from the substantia nigra of intact, GDX, and DHT supplemented male rats exposed to CIH. Proteins probed for were GFAP, an astrocyte marker (51kDa), NOX1 (49 kDa), and GAP-DH (37 kDa). Protein densitometries were normalized to GAP-DH densitometry readings for analysis. **B)** No significant differences in NOX1 expression were observed in the SN. **C)** GDX rats exhibited significantly higher astrocyte expression than gonadally intact rats. **D)** Representative western blots results from the entorhinal cortex of intact, GDX, and DHT supplemented male rats exposed to CIH. **E)** DHT administration resulted in significantly more NOX1 expression in the ETC than either intact or gonadectomized male rats. **F)** DHT rats exhibited significantly higher astrocyte expression than gonadally intact or GDX rats. **G)** Representative western blots results from the hippocampus of intact, GDX, and DHT supplemented male rats exposed to CIH. **H)** DHT administration resulted in significantly more NOX1 expression in the hippocampus than either intact or gonadectomized male rats. **I)** GDX rats exhibited significantly less astrocyte expression than gonadally intact or DHT rats. *Results are shown as mean ± s.e.m. $p < 0.05$; ** vs. intact, # vs. all other groups*

FIGURE 12

ANDROGEN ACTION ON CNS OXIDATIVE STRESS AND ASTROCYTES

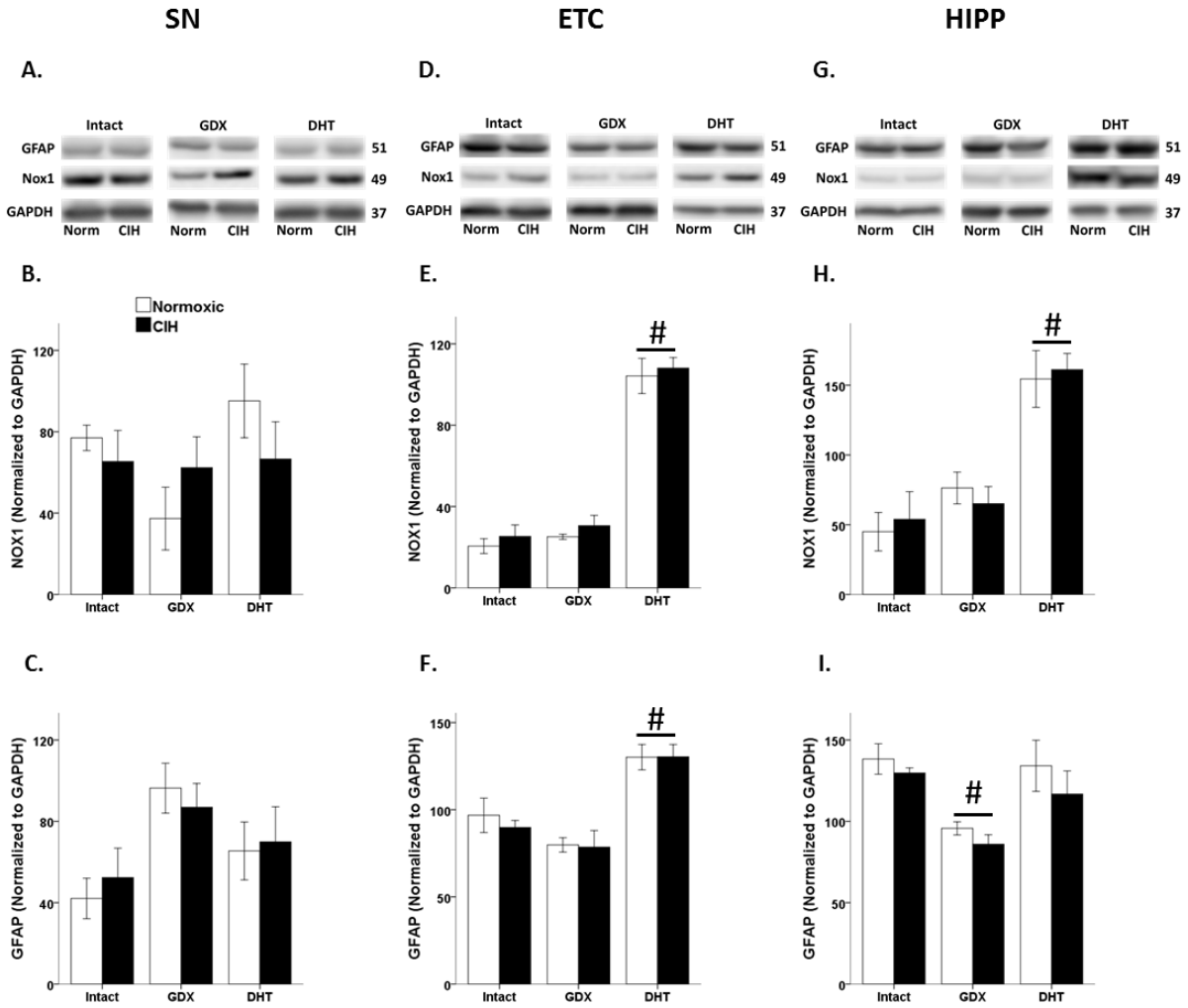

TABLE 6

CHRONIC INTERMITTENT HYPOXIA AND ANDROGENS INDUCE INFLAMMATION

		M1 cytokines			M2 cytokines		
		NORM	CIH		NORM	CIH	
Sample	hormone	mean ± s.d.	mean ± s.d.	*sig.*	mean ± s.d.	mean ± s.d.	*sig.*
plasma	INTACT	681.12 ± 305.41	534.08 ± 251.88		1119.08 ± 650.42	752.91 ± 331.77	
	GDX	236.95 ± 127.96	133.34 ± 49.27	**	232.17 ± 135.80	119.72 ± 78.20	#
	DHT	382.62 ± 82.12	581.64 ± 344.90		582.73 ± 121.17	547.71 ± 128.93	
substantia nigra	INTACT	17.80 ± 2.64	**20.99 ± 1.77**	*	10.02 ± 1.25	9.21 ± 3.78	
	GDX	21.40 ± 0.64	**23.35 ± 2.49**	*	7.62 ± 7.07	9.53 ± 6.51	
	DHT	32.64 ± 11.58	**45.59 ± 4.52**	*#	0.87 ± 1.10	1.70 ± 1.42	#
entorhinal cortex	INTACT	342.98 ± 182.03	426.48 ± 119.87		1201.90 ± 559.38	1474.81 ± 294.04	
	GDX	119.32 ± 6.26	92.62 ± 22.06	**	437.91 ± 60.47	258.76 ± 104.67	#
	DHT	174.73 ± 38.18	205.41 ± 57.09	**	724.82 ± 226.04	790.34 ± 194.39	#
hippocampus	INTACT	120.18 ± 21.83	**74.90 ± 19.61**	*	489.09 ± 129.74	**242.44 ± 105.62**	*
	GDX	117.51 ± 21.12	84.97 ± 35.66		481.61 ± 99.16	296.50 ± 238.95	
	DHT	81.25 ± 5.05	81.30 ± 21.01		267.63 ± 90.67	226.57 ± 73.19	

Table 6: comparison of average pro-inflammatory M1 cytokines (IL-1β, IL-6, TNF-α, and GM-CSF) and anti-inflammatory M2 cytokines (IL-4, IL-10, and IL-13) in hormone groups exposed to CIH or normoxic conditions. *$p < 0.05$; * numbers in bold font indicate differences due to CIH, ** compared to intact, # compared to all other groups*

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CHAPTER III

DISCUSSION

Androgens and Oxidative Stress

The investigation of interactions between oxidative stress and sex hormones is an active area of research with respect to estrogen signaling (4, 16). However, data on androgen signaling and oxidative stress interactions are sparse. Prior *in vitro* studies have shown that androgens, such as testosterone and dihydrotestosterone, can have either protective or damaging effects that are dependent on the level of oxidative stress in the environment (7). Whether these interactions occur within tissue composed of heterogeneous cell types is unknown. Furthermore, few studies have used animal models to examine the impact of androgens and oxidative stress in animal models with minimal oxidative stress induced cell death. We found that the sleep apnea animal model, chronic intermittent hypoxia (CIH), meets this requirement of increased oxidative stress and no significant loss of brain cells in young gonadally intact male rats (15).

Oxidative stress is a hallmark of neurodegenerative disorders (9). Mechanisms which contribute to the unchecked accumulation of oxidative stress are suspected to accelerate end organ damage and lead to disease onset (8). However, conditions which slightly elevate basal oxidative stress, such as moderate exercise, appear to upregulate expression of mitochondria and antioxidant systems within a cell, and provide pre-conditioning protection against later oxidative insults (11, 13).

Prior *in vitro* studies have shown testosterone is an oxidative stressor (7, 14). This appears to underlie its protective mechanisms, as oxidative stress insults in cell lines pre-treated with testosterone do not result in apoptosis (7, 14). Indeed, in the current study, male rats with testosterone had significantly elevated basal oxidative stress than hormone-deficient gonadectomized rats (figure 7). More importantly, the rats which received testosterone capsule implants and did not experience CIH-induced testosterone loss, were protected from CIH-induced oxidative stress, and exhibited no behavioral deficits. It appears that, similar to exercise, preventing hypothalamic-pituitary-gonadal (HPG) axis impairment by maintaining physiological levels of testosterone is neuroprotective.

It was not possible to determine the interactions between hormone deficient (gonadectomized) male rats and oxidative stress (induced by CIH), as gonadectomy resulted in significant memory impairments. Therefore, other avenues of investigation were necessary to determine the mechanisms of androgen action under an oxidative stress environment. Since testosterone has been reported as both protective and damaging in the CNS, it has been proposed that the neuroprotective effects of testosterone is due to its metabolite, estrogen (3, 5, 10, 12). Conversely, testosterone's non-aromatizable androgenic metabolite, dihydrotestosterone (DHT), is associated with damaging processes within neurons and appears to trigger different cognitive processes in the hippocampus (1, 2, 6). *In vitro* studies in our lab support a detrimental effect of DHT on oxidative stressed neurons. Therefore, we used DHT to investigate the non-aromatizable effects of testosterone in an oxidative stress environment. DHT in an oxidative stress environment (induced by CIH) impaired memory retention and further exacerbated oxidative stress generation and inflammation in brain regions associated with neurodegenerative disease, such as the substantia nigra, entorhinal cortex, and hippocampus (Figure 13).

FIGURE 13

PROPOSED INTERACTION BETWEEN ANDROGENS AND OXIATIVE STRESSORS ON NEURONS AND ASTROCYTES

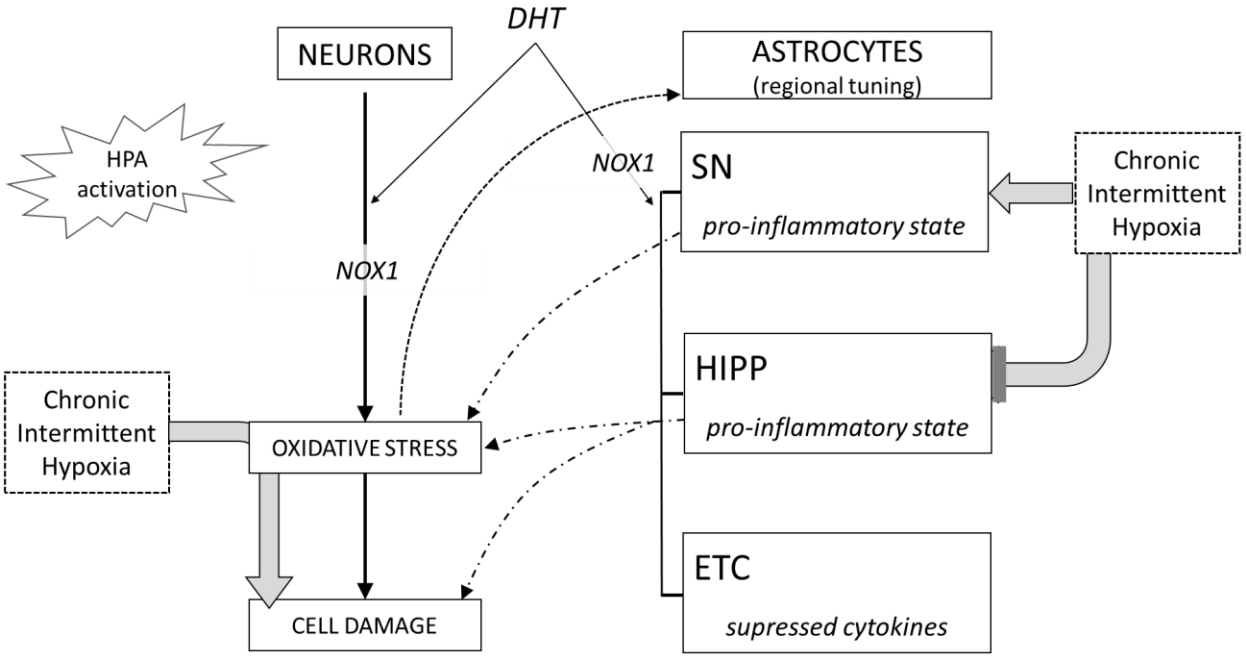

Figure 13 Astrocytes are the innate immune source for inflammatory cytokine production in the brain. Chronic intermittent hypoxia (CIH) increases oxidative stress and pro-inflammatory pathways. Dihydrotestosterone (DHT) elevates NOX1 expression, leading to an increase in oxidative stress and immune response. In the presence of CIH, DHT exacerbates damage and memory deficits. Hypothalamus-Pituitary-Adrenal (HPA) activation can serve to amplify effects of oxidative stressors.

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RAT STRAIN AND HOUSING CONDITIONS ALTER OXIDATIVE STRESS AND HORMONE RESPONSES TO CHRONIC INTERMITTENT HYPOXIA

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* *to be submitted to Brain Research*

Abstract

Sleep apnea has been associated with elevated risk for metabolic and cardiovascular disorders. Further, the role of hypothalamic-pituitary-adrenal (HPA) activation in sleep apnea has been controversial in human studies. Chronic intermittent hypoxia (CIH) is a rodent model which mimics the hypoxic events experienced by patients with sleep apnea. Most studies of CIH in rats have been conducted in the Sprague-Dawley rat strain. Previously published literature suggests different strains of rat exhibit various responses to disease models, and these effects can be further modulated by the housing conditions experienced by each strain. This variability in response is similar to what has been observed in clinical populations, especially with respect to the HPA system. To investigate if strain or housing (individual or pair-housed) can affect the results of CIH (AHI = 8) treatment, we exposed individual and pair-housed Sprague-Dawley and Long-Evans male rats to 7 days of CIH treatment. This was followed by biochemical analysis of circulating hormones and oxidative stress markers. Both strain and housing conditions altered oxidative stress generation, corticosterone (CORT) and adrenocorticotropic hormone (ACTH), and weight metrics. Specifically, pair-housed Long-Evans rats were the most sensitive to 7-day CIH, which showed a significant association between oxidative stress generation and HPA activation. These results suggest both strain and housing conditions can affect the outcomes of CIH, especially the HPA system.

Background

There is a lack of consensus in the literature related to the basic scientific model of sleep apnea (SA), chronic intermittent hypoxia (CIH). CIH has been reported to be both protective and damaging in subsequent stroke outcomes Elevated mean arterial pressure, inflammation, and oxidative stress have been measured in models of CIH (6, 31, 37, 40), while other studies have reported lower oxidative stress and pre-conditioning effects of CIH (47, 49). These divergent reports further complicate interpretation of the CIH animal model and consequently our understanding of SA (27). The pivotal factors contributing to these dichotomous observations of CIH appear to be the frequency and severity of the intermittent hypoxia used in each study. There appears to be a threshold in which studies using a more frequent normal room air to low oxygen cycle per hour result in damaging effects, while models with very slow air changes per hour report protective mechanisms.

In addition to differences in CIH protocols, prior studies have been conducted on various rat strains under different housing conditions. Our laboratory has exposed single-housed Sprague-Dawley (40) and pair-housed Long-Evans (38, 39) rat strains to CIH with varying responses to oxidative stress. Most CIH protocols have been performed on either Sprague-Dawley or Wistar rat strains and not the Long-Evans rat strain. Generally, housing conditions are not reported (7, 24, 29, 45). Of the studies that include housing conditions, it is interesting to note that publications using single-housed Sprague-Dawley rats report increased oxidative stress in response to CIH (37, 40). Therefore, rat strain and housing conditions may be important variables.

The scientific community has recognized a number of factors exist which contribute to contradictory reports in many basic science models. In recognition of this, the National Institutes

of Health (NIH) updated their mission of Rigor and Responsibility to improve reproducibility (19). Current variables recognized as having an impact on experimental outcomes are sex, age, weight, and current health status. Here, we present results supporting model strain and housing conditions as key components affecting the observed outcomes in our study using CIH as an oxidative stressor. These results suggest inclusion of these two variables should be included in future studies.

Materials and Methods

Animals: Two out-bred rat strains were used in this study – Sprague-Dawley and Long-Evans. Adult male Sprague-Dawley and Long-Evans rats (both strains 58-64 days old, 250-275 g body weight, Charles River) were housed in a temperature controlled environment with the lights on a 12:12 hour cycle. The Sprague-Dawley rat strain is a non-aggressive rat strain, whereas the Long-Evans rat strain is more aggressive and active than Sprague-Dawley rats (2, 18, 44). Thus, we examined single-housing versus pair-housing in both rat strains. Upon arrival, animals were either housed individually or pair-housed for the remainder of the experiment. Food and water were provided *ad libitum.* Animals were weighed each week during cage cleaning and at the end of testing. All experiments were conducted according to National Institute of Health guidelines on laboratory animals and approved by the Institutional Care and Use Committee at UNT Health Science Center.

Chronic Intermittent Hypoxia (CIH): Hypoxic A-Chambers and OxyCycler A84XOV controllers were purchased from Biospherix, Ltd. (Parrish, NY). One week after arrival, rats were separated into either normoxic or CIH treatment groups with at least 10 animals per group.

This resulted in 8 treatment groups: single-housed Sprague-Dawley normoxic (n = 10), single-housed Sprague-Dawley CIH (n = 10), pair-housed Sprague-Dawley normoxic (n = 17), pair-housed Sprague-Dawley CIH (n = 16), single-housed Long-Evans normoxic (n = 12), single-housed Long-Evans CIH (n = 12), pair-housed Long-Evans normoxic (n = 12), and pair-housed Long-Evans CIH (n = 12). Home cages, each containing either single or pair-housed animals, were placed into each A-chamber for acclimation to the apparatus for one week at normoxic conditions (21% oxygen). Acclimation to the chambers was followed by CIH exposure for 7 days from 8 am to 4 pm during the light (sleep) phase. Our CIH protocol utilized 8 minute cycles of low oxygen (10%) followed by reoxygenation (21%) for 8 hours during the light phase to model an AHI = 8 (14, 39, 46). Specifically, nitrogen was injected into the chamber over a period of 5 minutes to reach a low oxygen concentration of 10%, followed by injection of oxygen over 3 minutes to return to and maintain normal room air concentrations (21%). For the remaining 16 hours, animals were exposed to room air. To control for sleep deprivation, due to noises from the CIH apparatus, normoxic controls were housed under similar conditions but not administered hypoxia.

Sample Collection: Between 0800 and 1000 on the morning following the final CIH exposure, which was during the first two hours of the light phase, animals were anesthetized with isofluorane (2-3%) and sacrificed by decapitation, as previously described (40). Blood was collected in 7 mL EDTA tubes. The samples were then centrifuged at 2,000 x g for 10 min at 4°C. Plasma was removed and aliquoted for storage in microcentrifuge tubes at -80°C until assayed.

Advanced Oxidative Protein Products (AOPP) assay: Circulating oxidative stress was assayed using Cell Biolabs, Inc. OxiSelect Advanced Oxidative Protein Products assay kit, according to our previously published protocol (11, 39, 40). This kit measures the amount (uM) of all oxidized proteins in the sample relative to a known standard. Chloramine in the kit reacts with oxidized proteins to produce a color change which can be read at 340nm. Assay results were reported as percent of control (individual value / (average of control values) x 100).

Hormone measurements:

Circulating nadir corticosterone (CORT) was assayed using a commercially available competitive immunoassay (Corticosterone Mouse/Rat ELISA kit, BioVendor), according to manufacturer's instructions. Sensitivity of the assay was 6.1 ng/ml at the 2 s.d. confidence limit. The intra-assay coefficient of variation was 7.37% and the inter-assay coefficient of variation was 7.63%. Specificity of this assay is as follows: corticosterone (100%), cortisol (2.3%), aldosterone (0.3%), testosterone (< 0.1%), progesterone (6.2%), and androsterone (< 0.1%). Results are expressed as ng/ml.

Plasma adrenocorticotropic hormone (ACTH) was assayed by double-antibody radioimmunoassay using ^{125}I (MP Biomedicals, Solon, OH, USA), according to manufacturer's protocol. Samples were performed in duplicates and the assay was measured using a gamma counter (Cobra Auto-Gamma, LPS Biomedical Instrument Services, Redmond, WA, USA), with counting time of 3 minutes per sample at 80% efficiency. The intra-assay coefficient of variation was 5.45% and the inter-assay coefficient of variation was 7.30%. The specificity of the assay is as follows: $ACTH^{1-39}$ (100%), $ACTH^{1-24}$ (100%), hβ Lipotropin (0.8%), hα Lipotropin (0.1%), hβ

Endorphin (<0.1%), hα MSH (<0.1%), and hβ MSH (<0.1%). The following formula was used to determine the %B/B0:

$$((CPM_{Sanple} - CPM_{NSB}) / (CPM_{0\ Standard} - CPM_{NSB})) \times 100$$

in which CPM = Counts per Minute, NSB = Non-specific binding (Blank), 0 Standard = Total binding (B0). The %B/B0 for the unknowns were then plotted against the standard %B/B0 using a 4 parameter-log function to determine ACTH concentration (analysis on myassays.com). Results are expressed as ng/ml.

Statistical analysis: IBM SPSS (SPSS v. 23, IBM, 2015) was used for statistical analysis. 3-way ANOVA was used to test for significant interactions between strain, housing condition, and hypoxic exposure. Fisher's LSD was used for post-hoc analysis. Results are shown as mean ± SEM. Statistical significance for all measurements was at $p \leq 0.05$.

Results

Previously, we have published that exposure to CIH (AHI = 10) induces elevated oxidative stress in single-housed Sprague-Dawley male rats (40). To investigate if this observation is maintained across strain and housing conditions, both Sprague-Dawley and Long-Evans male rats were housed singly or in pairs and then exposed to 7 days mild CIH (AHI = 8) or normoxic conditions. ANOVA indicated a significant interaction between strain, housing, and hypoxia ($F_{1,89} = 5.842$; $p < 0.05$). A significant elevation of oxidative stress was observed in pair-housed Long-Evans rats (139.84 ± 32.87 %) (figure 14) following CIH exposure. Unlike our previous observations, single-housed Sprague-Dawley rats did not have a significant increase in oxidative stress due to CIH at AHI = 8. Interestingly, pair-housed Sprague-Dawley rats and single-housed Long-Evans rats did not experience an increase in oxidative stress following 7

days of CIH. This suggests housing conditions impacts susceptibility to oxidative stress differently between strains.

Corticosterone (CORT), and its releasing hormone, ACTH, can be affected by housing conditions, and CORT may contribute to oxidative stress burden (41, 42, 48). To determine if strain type or housing conditions may produce a differential response to mild CIH in these parameters, circulating nadir CORT and ACTH were assayed. A significant interaction between strain and CIH ($F_{1,63} = 5.988$, $p < 0.05$) was observed in ACTH levels, with a main effect of both strain ($F_{1,63} = 75.735$, $p < 0.05$) and CIH ($F_{1,63} = 13.475$, $p < 0.05$) on plasma ACTH levels (figure 15a). Long-Evans rats had significantly higher ACTH (326.39 ± 108.14 ng/ml) than Sprague-Dawley rats (165.90 ± 55.13 ng/ml). In pair-housed Long-Evans rats, CIH significantly depressed ACTH (248.79 ± 63.25 ng/ml) compared to normoxic ACTH levels (377.79 ± 91.37 ng/ml).

A main effect of CIH on CORT was also observed ($F_{1,61} = 4.481$, $p < 0.05$). Similar to ACTH, Long-Evans pair-housed rats exposed to CIH had significantly lower CORT (26.04 ± 16.82 ng/ml) than their normoxic counterparts (50.64 ± 11.82 ng/ml) (figure 15b). Since prior studies have shown that CORT can increase oxidative stress (42, 48), we wanted to determine if there was a relationship between CORT and CIH-induced oxidative stress. Therefore, we investigated this association on our treatment groups (single-housed Sprague-Dawley and pair-housed Long-Evans rats) that showed an elevation of oxidative stress in response to CIH. We observed a positive association between CORT and oxidative stress only in Long-Evans pair-housed rats exposed to CIH (figure 16a), but not in Sprague-Dawley single-housed rats (figure 16b). This indicates there is a difference in sensitivity to an oxidative stressor that is dependent on strain and housing.

Activation of the HPA system can influence body weight (4, 16, 28). In our study, a statistically significant interaction between strain and housing conditions on weight was observed ($F_{1,93} = 7.597$; $p < 0.05$). Analysis of the main effects revealed a significant difference in weight between the two strains of rats ($F_{1,93} = 41.746$, $p < 0.05$), with Long-Evans rats weighing more (389.77 ± 46.75 g) than Sprague-Dawley rats (343.09 ± 26.05 g) (figure 17). A significant difference in housing ($F_{1,93} = 5.075$, $p < 0.05$) was observed in Long-Evans rats in which pair-housed Long-Evans rats weighed less (371.50 ± 24.88 g) than the single-housed Long-Evans rats (408.04 ± 56.12 g). The housing conditions of Sprague-Dawley rats did not affect weight in this study. Additionally, exposure to CIH did not affect the final weight of any of the treatment groups or correlate with oxidative stress measurements (Table 7).

Discussion

Our current experiment utilized a protocol modeling an AHI = 8 to examine the effects of mild CIH treatment on two different strains of rats, Sprague-Dawley and Long-Evans, housed either singly or in pairs. Behavioral studies have provided evidence that strain differences and social interaction via housing conditions can affect the outcome of many studies (5, 15, 16, 23, 43, 44). Consideration of these factors allows for a more robust understanding of the mechanisms of disease and improves therapeutic outcomes.

Interestingly, only one study has examined the differences between Sprague-Dawley and Long-Evans rats in response to oxygen exposure. Unlike our study using intermittent low oxygen levels, Chrysostomou *et.al.* examined hyperoxia (75% oxygen) exposure for 14 days and found Long-Evans rats were more sensitive to oxygen than Sprague-Dawley rats, resulting in increased cell death and astrocyte upregulation (10). Consistent with these observations, significant

differences in oxidative stress in a strain dependent manner were observed in this study (figure 14). Pair-housed Long-Evans rats exhibited an increase in oxidative stress similar to what was previously observed in the single-housed Sprague-Dawley rats at a slightly higher AHI (40). The single-housed Sprague-Dawley rats used in this study did not show significant increase in oxidative stress under CIH conditions. Thus, Long-Evans rats in group housing conditions may be more sensitive to oxidative insults than Sprague-Dawley rats. Interestingly, neither the pair-housed Sprague-Dawley nor the single-housed Long-Evans rats exhibited an oxidative stress response to early mild CIH. These results suggest an interaction between genetic differences in the rat strains and housing conditions influences oxidative stress and HPA system. These parameters should be considered when investigating mechanisms contributing to oxidative stress.

In both humans and rodents, social interaction and environmental stress impact disease risk (33). Activation of the hypothalamus-pituitary-adrenal (HPA) axis occurs under stressful scenarios. Elevated CORT or ACTH is indicative of a HPA activation, and is necessary in maintaining sleep architecture (21, 26). CORT and ACTH fluctuate in diurnal patterns that mirror each other (8, 9). We observed differences in nadir ACTH between strains that are consistent with literature (figure 16). Although previous publications have reported a difference in CORT between Sprague-Dawley and Long-Evans rats, there were no basal differences in CORT due to strain or housing in this study. Hormones fluctuate diurnally, with low concentration occurring at the beginning of the sleep phase. Samples in this study were collected within two hours of lights on, so differences may not be observable at this time due to a floor effect. The difference in ACTH, in which Long-Evans rats have higher ACTH than Sprague-Dawley rats, suggests our measurements of the HPA axis are in agreement with existing publications (16, 25, 32).

Regardless, CORT levels were significantly negatively correlated with oxidative stress in the Long-Evans pair-housed rats, which exhibited a significant rise in oxidative stress due to CIH. Similar to our results, prior studies found basal ACTH was not altered in single-housed Sprague-Dawley rats exposed to 7 days CIH (AHI =10), but was more reactive when those same rats were subjected to a subsequent stressor (30 min immobilization). Sprague-Dawley rats housed individually consistently present significantly greater HPA responses than Sprague-Dawley rats in group housing, suggesting socialization desensitizes Sprague-Dawley rats to stress (36).

The 2011 recommendation by the National Research Council for the care and use of laboratory animals is that social animals, such as rats, are to be housed in pairs or as a group (1). This recommendation was based on prior studies using Sprague-Dawley and Wistar rat strains that found decreased stress responses in rats housed in groups of 3-4 male rats/cage (22, 36). Similarly, our results show that housing conditions did not adversely affect Sprague-Dawley male rats. No differences in HPA hormones or body weights were found in either individual housed or pair-housed, consistent with prior reports in Sprague-Dawley male rats (44). However, not all rat strains respond the same to housing conditions. Group housing in Long-Evans rats has been associated with increased anxiety and reduced body weight (3, 34). Similarly, our results showed male Long-Evans rats were adversely affected by pair housing, as evidenced by corticosterone and ACTH impairments and decreased body weights. It has been proposed that this strain difference could be due to the level of aggression displayed by the different rat strains. For example, the Sprague-Dawley rat strain is a non-aggressive rat strain, whereas the Long-Evans rat strain is aggressive (2, 18). Henry et. al. found that group housing of unfamiliar adult Long-Evans rats, and not Sprague-Dawley rats, resulted in a prolonged activation of the stress-

response and the inability to establish a stable dominance hierarchy (17, 18). Indeed, we observed increased aggressive behaviors (attacks, threats, aggressive mounts, boxing, and dominance postures (12)) by Long-Evans males and not in Sprague-Dawley males (data not shown). Therefore, this activation of the HPA axis may underlie the observed differences in Sprague-Dawley and Long-Evans rat strains to CIH.

These results suggest that mechanisms which render an organism susceptible to impairment of the HPA axis may also confer susceptibility to oxidative stress insults. The results may not be immediately observable under non-stressful conditions, but manifest with the addition of a psychological or physiological stressor. A similar phenomenon is observed in clinical populations who experience chronic life stressors or illness and are subsequently exposed to an additional injury or infection (13, 20, 30, 35). They often succumb more rapidly and have lingering health concerns compared to individuals with less stress-response activation. Therefore, SA mechanisms may be additive and pose the highest risk to individuals with additional physiological or psychological stress.

Differences in strain response to CIH were observed in oxidative stress and corticosterone/ACTH measurements under different housing conditions. These results underscore the need of housing conditions to be included with strain reporting, especially in the investigations of any stressful stimuli, such as CIH, as factors which affect the HPA axis may influence the outcome. This study may shed light on discrepancies found between labs that use different animal strains and housing conditions, as well as guide future experimental design choices when selecting an animal model.

Acknowledgements

The authors would like to thank Drs. J. Thomas Cunningham, Steve Mifflin, Ann Schreihofer, and Robert Luedtke for their advice and provision of experimental equipment. In addition, we would like to acknowledge the technical assistance of Joel Little, E. Nicole Wilson, and Jessica Proulx. This work was supported by grants from the NIH R01 NS091359 and The Alzheimer's Association New Investigator Research Grant NIRG-14-321722 to RLC and a NIH training grant T32 AG 020494 to BDS.

FIGURE 14

STRAIN AND HOUSING AFFECT CHRONIC INTERMITTENT HYPOXIA INDUCED OXIDATIVE STRESS

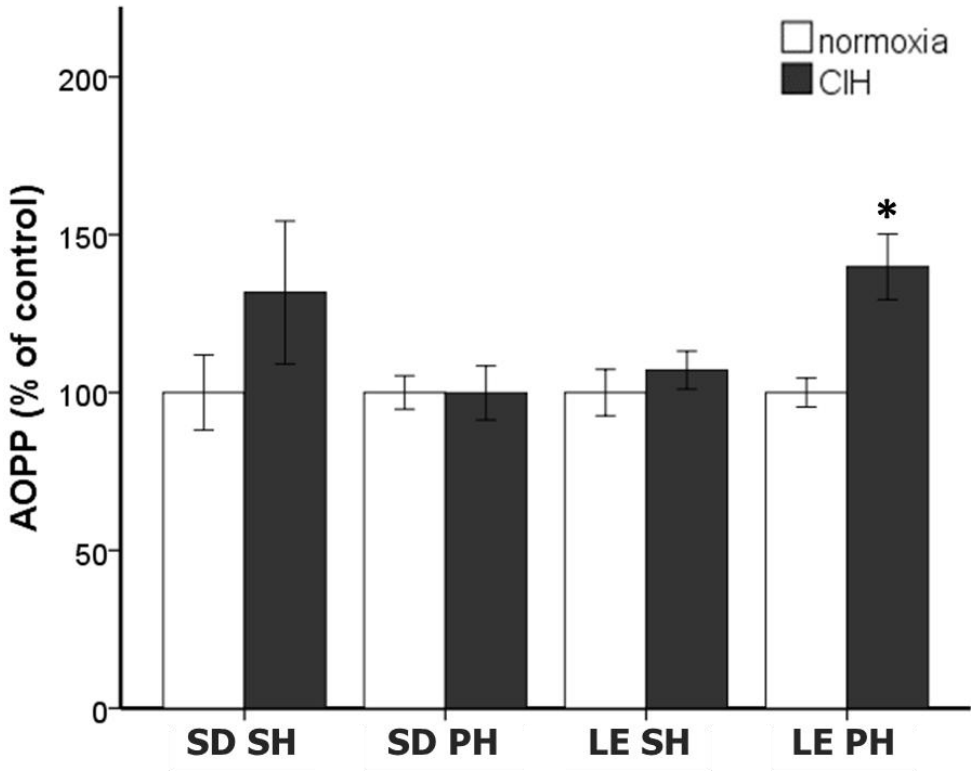

Figure 14: **Strain and housing conditions alter oxidative stress response to chronic intermittent hypoxia (CIH)** Oxidative stress was measured in plasma using Advanced Oxidative Protein Products (AOPP) assay from Cell Biolabs. Long-Evans pair-housed rats (LE PH) exhibit significantly higher oxidative stress when exposed to chronic intermittent hypoxia than normoxic controls. In Sprague-Dawley single-housed rats (SD SH), oxidative stress was not increased by CIH. No significant differences in oxidative stress were observed in Sprague-Dawley pair-housed (SD PH) or Long-Evans single-housed (LE SH) rats. *Results are reported as mean ± s.e.m. (percent of normoxic control values), * compared to normoxic control; statistical significance was set at* $p \leq 0.05$.

Figure 15: **CIH impairment of the hypothalamic-pituitary-adrenal (HPA) axis is affected by rat strain and housing conditions.** A) Sprague-Dawley rats have lower circulating adrenocorticotrophic hormone (ACTH) than Long-Evans rats, which was not affected by housing or CIH. CIH significantly reduced circulating nadir ACTH in Long-Evans pair-housed (LE PH) rats, but not Long-Evans single-housed (LE SH) rats. B) No differences due to strain or housing were observed in circulating corticosterone (CORT), but CIH significantly decreased in CORT in Long-Evans pair-housed rats. *Results are reported as mean ± s.e.m. (ng/ml), * compared to normoxic control, # compared to Sprague-Dawley; statistical significance was set at* $p \leq 0.05$

FIGURE 15

STRAIN AND HOUSING CONDITIONS AFFECT THE HPA RESONSE TO CHRONIC INTERMITTENT HYPOXIA

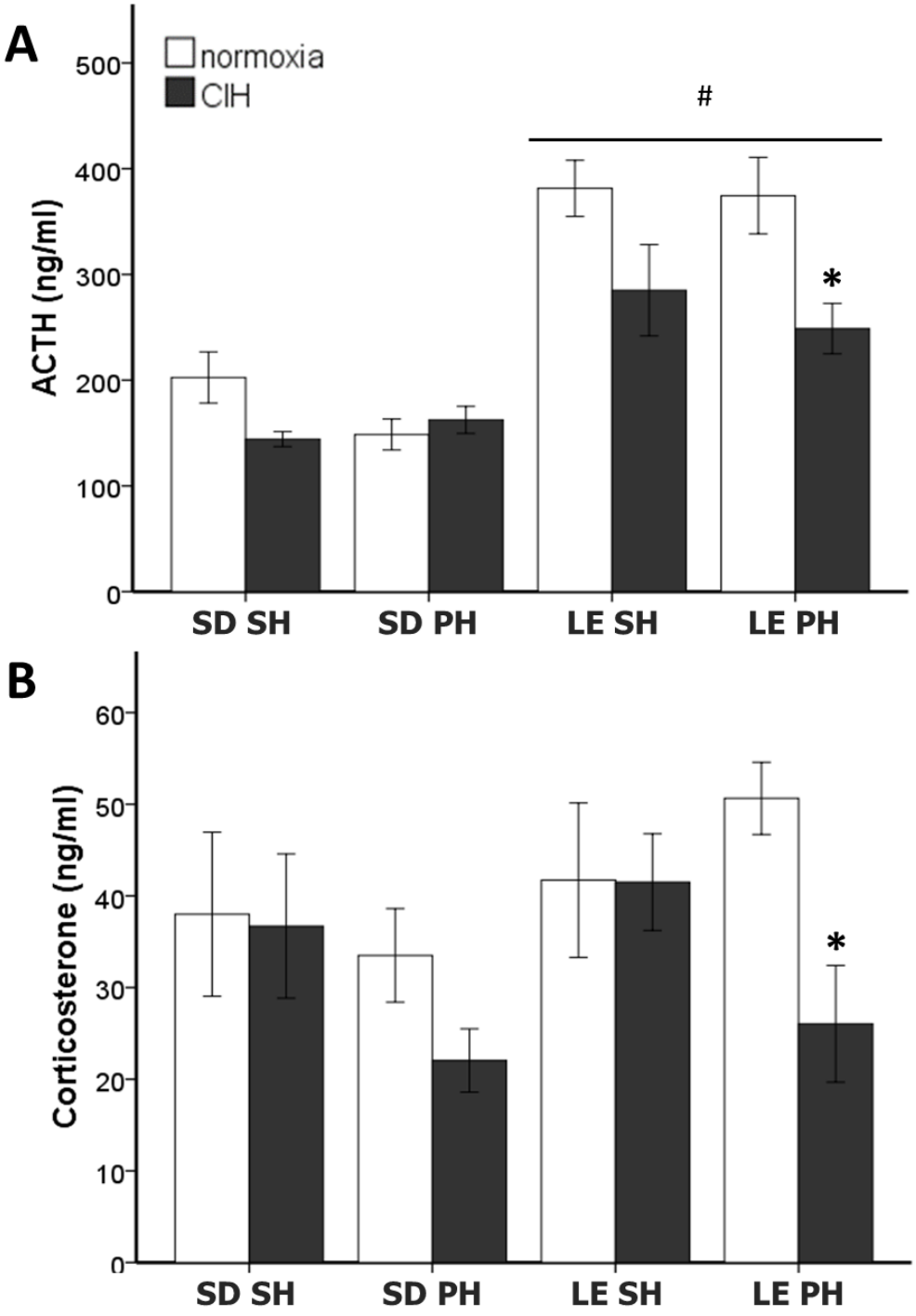

Figure 16: **Strain differences are apparent in the positive association of corticosterone and oxidative stress under CIH.** A) A significant positive correlation between CORT and AOPP was observed in Long-Evans pair-housed male rats following 7 days of CIH exposure at AHI =8. B) AOPP in Sprague-Dawley single-housed male rats not associated with CORT ($p = 0.129$). *Statistical significance was set at $p \leq 0.05$*

FIGURE 16

OXIDATIVE STRESS ASSOCIATION WITH OXIDATIVE STRESS UNDER CIH

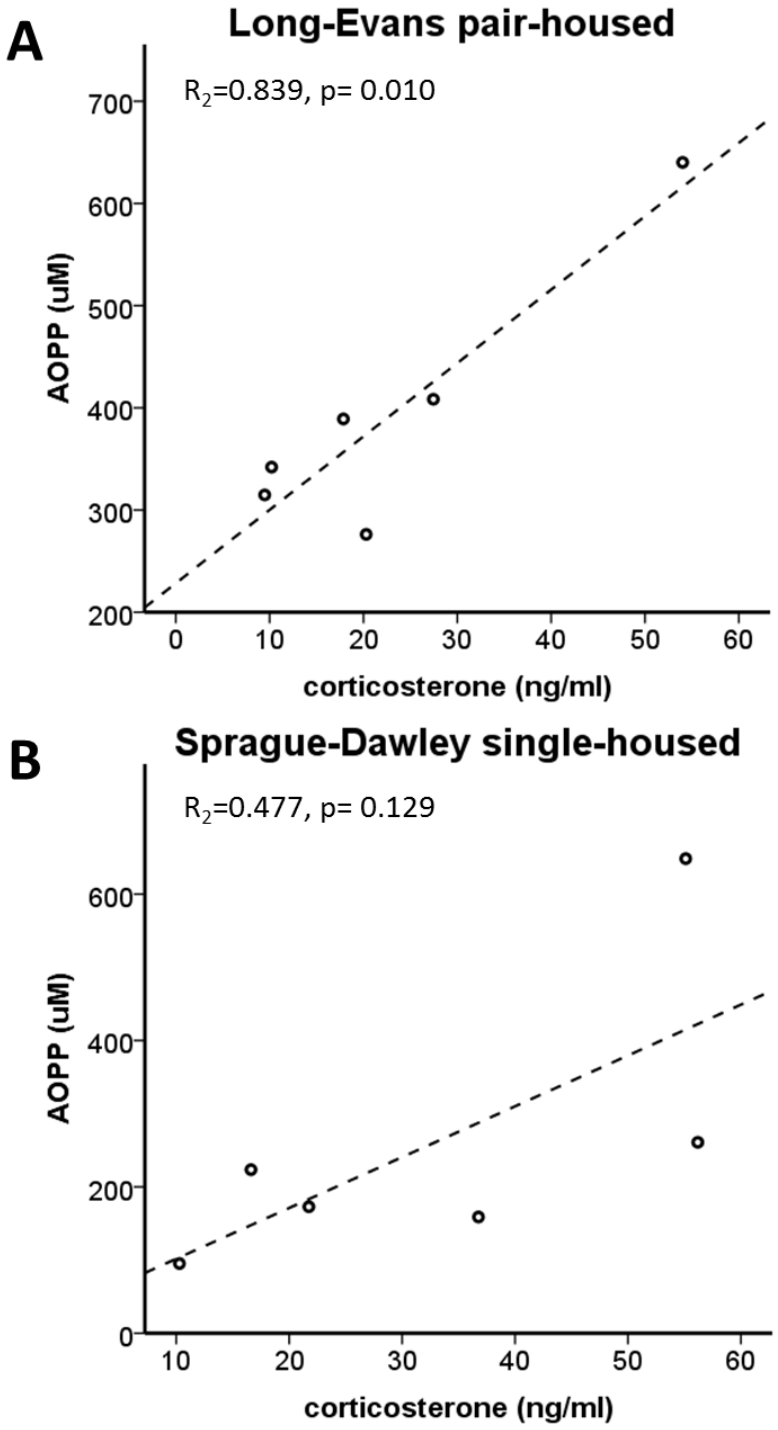

FIGURE 17

HPA AXIS CONTRIBUTES TO WEIGHT

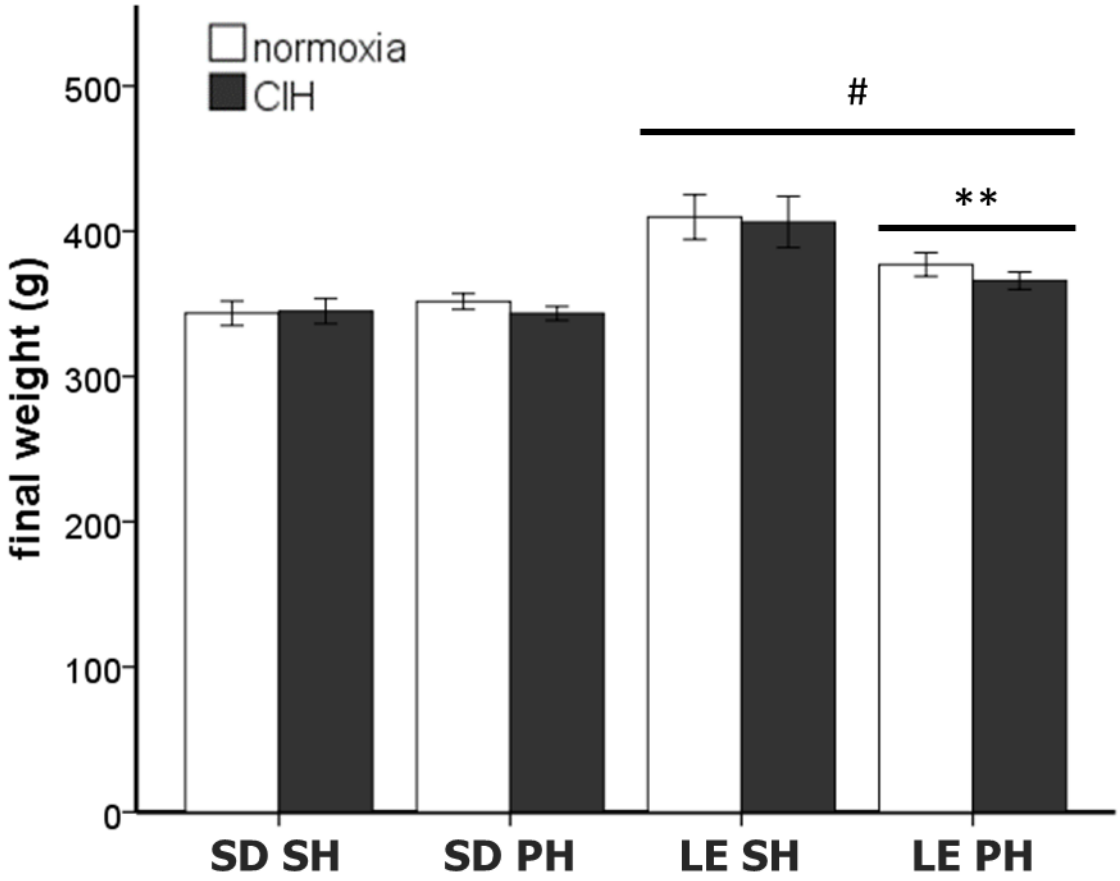

Figure 17: **Differences in weight are not due to chronic intermittent hypoxia.** Long-Evans rats were significantly heavier than Sprague Dawley rats, but no differences due to CIH were observed. *Results are reported as mean ± s.e.m. (g), # compared to Sprague Dawley strain, ** compared to single-housed; statistical significance was set at $p < 0.05$*

TABLE 7

WEIGHT (g) v. AOPP (uM)

strain	housing	hypoxia	r^2	*p-value*
Sprague-Dawley	single	norm	0.080	*0.43*
		CIH	0.124	*0.39*
	pair	norm	0.006	*0.77*
		CIH	0.008	*0.75*
Long-Evans	single	norm	0.211	*0.13*
		CIH	0.022	*0.65*
	pair	norm	0.283	*0.07*
		CIH	0.171	*0.24*

Table 7 Weight is not significantly associated with oxidative stress in either Sprague-Dawley or Long-Evans rats.

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Chronic Intermittent Hypoxia Model

Chronic intermittent hypoxia (CIH) has been developed as a model of the repetitive hypoxic events experienced by patients with sleep apnea (14, 34, 36). In this model, rats are placed into a chamber which allows for control of ambient oxygen levels. During the rat's sleep phase, nitrogen is infused into the chamber to lower the oxygen concentration, mimicking the drop in oxygen saturation observed in sleep apnea. This is followed by infusion of room air or oxygen to return the ambient oxygen concentration to normal levels of 21%. These cycles occur rapidly and repetitively over the course of 8 hours and result in rapid changes in blood oxygen saturation, similar to clinical populations. The duration of the air exchanges can be altered to model the desired apnea/hypopnea index (AHI) of interest.

In these studies, two CIH protocols were used in two different rat strains, under two different light cycles. The study by Snyder, et al. was conducted in Sprague-Dawley rats in custom built hypoxic chamber. In this study, nitrogen was infused into each chamber for 90 seconds, and held at 10% oxygen for an additional 90 seconds (43) under the normal light cycle (12:12, lights on at 0745). This was followed by room air infusion into the chamber for 90 seconds to return the concentration to 21% and was held at that point for an additional 90 sec. This modeled a mild AHI of 10 events per hour. In the androgen study, Long-Evans rats were used, and the AHI was lowered to 7.5 – 8 events per hour in a commercially available system. In this protocol, oxygen concentration was reduced to 10% over the course of 5 minutes and returned to normal concentrations within 3 minutes. Additionally, the light phase was reversed

from 0700-1900 to 2100-0900. The study comparing the two rat strains utilized the same cycle as the androgen study, but during normal light cycles used in the Snyder, et al study.

The selection of each CIH protocol was influenced by factors unique to each study. The Snyder, et al. study established the rise of oxidative stress under the same protocol which has been used by prior studies to examine hypertension induced by CIH. This validated the use of CIH as a clinically relevant model of a chronic disease which can induce oxidative stress in brain regions affected by neurodegeneration.

In the androgen study, the use of a different rat strain, a change in housing conditions, and the addition of hormones, necessitated modifications to the initial protocol. A major focus of this project was the early effect of CIH on memory and motor behavior. To this end, the Long-Evans rat strain was selected, which has been characterized as more active than the Sprague-Dawley rat strain used in the initial study (47). To avoid additional disruption of sleep during behavioral assessments, the rats' light phase was reversed upon arrival in the facility and maintained throughout the duration of the experiment. In addition, a requirement to pair-house our animals, rather than individually house them, resulted in cages which were too large to fit into the original CIH apparatus. A commercially available system was obtained for the remainder of the experiments to accommodate the new cage sizes. It was hypothesized the addition of androgens would exacerbate the oxidative stress generated by CIH. This raised the concern of apoptosis occurring within the brain regions of interest, which was not desirable for purposes of this study. Therefore, the cycles were extended to reduce to AHI to a milder application.

The rat strain study was conducted to verify the lower AHI induced the same effects in the Sprague-Dawley strain as the original protocol, as well as to compare the effects of housing

and strain selection. In this study, the lower AHI was used in the same commercially available chambers, although the light phase was not shifted.

Within each study, sample collection occurred between 0800 -1100, which resulted in different periods of the circadian cycle being studied for those animals under reverse light conditions compared to normal light conditions. Similar effects of CIH on oxidative stress, which is a cumulative measure, was observed in each study (figures 2, 8, 14). However, the timing of collection may have played a role on the results of biomarkers which are known to cycle diurnally. Inflammation, glial cells, corticosterone, and ACTH concentrations fluctuate throughout the day, with concentrations peaking at characteristic points (8, 23, 42). For example, inflammatory cytokines peak late during the active phase and drop to nadir by the end of the sleep phase. Similarly, glial cells are more reactive at the same time points, while corticosterone and ACTH peak late during the sleep phase to initiate waking. These expression patterns may partially explain some of the differences observed between the studies and should be carefully considered for sample collection time-points in future experimental designs.

Other Contributors to Oxidative Stress

In addition to rat strain and housing conditions influencing interactions between testosterone and oxidative stress, we found evidence HPA and circadian rhythms can influence these interactions. Gonadal and adrenal circulating hormones fluctuate in characteristic diurnal patterns (8). These patterns are associated with maintaining healthy sleep architecture necessary for cell and energy regeneration and memory acquisition (19). Scenarios which cause disruption in bioavailable hormones lead to changes in sleep structure, such as extension/reduction of time spent in REM or deeper phases of sleep (3, 13, 16, 20, 22, 27). Alteration of sleep structure itself

causes a disruption in circulating hormones, creating a problematic cycle, which may center around oxidative stress (29, 33). Unlike our previous studies that found elevated peak (during early wake stage) corticosterone levels in response to CIH (50), our results found an impaired (suppressed) stress hormone response (circulating ACTH and corticosterone) in plasma samples from pair-housed Long-Evans rats obtained during the sleep cycle. Furthermore, this suppressed corticosterone was significantly associated with CIH-induced oxidative stress, indicating that the impaired stress response was involved in oxidative stress generation (figure 16). This supports the need for further investigation into the role of the circadian rhythm on oxidative stress and steroid hormone mechanism(s) (figure 13). It has been well-established that significant changes in circadian rhythms over time leads to adverse health consequences, including increased risk for Parkinson's disease and Alzheimer's disease (6, 19, 28, 48).

Translational Impact

The fact that increased oxidative stress, changes in HPG and HPA hormones, and memory impairments are all observable after only one-week exposure to an oxidative stressor (CIH at low AHIs) has serious implications for clinical populations with sleep apnea and other oxidative stress-related pathologies. Most cases of sleep apnea go undiagnosed (18, 53). Upon diagnosis, treatment of sleep apnea is only deemed necessary for severe conditions (AHI < 30). Patients with moderate sleep apnea (AHI 15-30) are recommended to receive treatment, and treatment of mild sleep apnea (AHI < 15) is considered optional (12, 21). Additionally, patient compliance with CPAP treatment is low and patients often halt treatment within 1 year (51). This implies patients may be at risk for comorbid neurodegeneration, cardiovascular disease, and metabolic disorders, as well as HPG and HPA impairments, well before they are diagnosed with

sleep apnea, and this risk may continue unchecked post-diagnosis. Small clinical studies provide promising evidence that treating sleep apnea may prevent the onset of neurodegeneration (2, 10, 35, 46). Longitudinal studies with larger cohorts including all stages of sleep apnea severity are necessary to fully determine the effectiveness of sleep apnea treatment on comorbid disease prevention. It may be prudent for sleep assessment to be a routine component of risk assessment for patients who present with hypertension, elevated oxidative stress, or hormone irregularities. Clinical sleep assessments can be time and financially prohibitive, but recent development of small monitoring devices which can be utilized at home may prove useful tools for clinicians to assess at-risk patients and target effective therapeutics (31).

Of further concern is the fairly recent acknowledgement by the National Institutes of Health (NIH) that women respond differently to many diseases and therapies and are underrepresented in most medical studies (1). Many of their differences may be attributable to sex hormone composition, as estrogen, not testosterone, is the primary circulating hormone for women (4, 37, 45). However, just as men are responsive to estrogens, women also have circulating androgens and are subject to its effects. Women are diagnosed with sleep apnea less frequently than men are, but this may be due to different symptom presentation which often results in depressive disorders instead (5). In addition, women with sleep apnea are more frequently diagnosed with mild sleep apnea, suggesting they are at particular risk to neurodegenerative processes initiated by untreated mild sleep apnea (25). Women experience a more drastic change in their primary sex hormone, estrogen, than men do in testosterone as they go through menopause. This results in a relative increase in androgenic to estrogenic signaling as women age. Their health risk for sleep apnea onset, neurodegeneration, cardiovascular disease, and metabolic disorders all rise dramatically during post-menopause, suggesting the loss of

estrogen represents a loss of protection (4, 9, 41, 45). Indeed, women with polycystic ovarian syndrome have uncharacteristically high circulating androgens throughout their reproductive years, and this is associated with an increased incidence of sleep apnea, hypertension, insulin insensitivity, and neurodegenerative risk with age (7, 15, 39, 40, 52). Androgens clearly play a role in women's sleep apnea associated comorbid disorders and should be further investigated as the off-label use of testosterone in women has risen over recent years (17).

Future Studies

One of the limitations of using the CIH animal model as an oxidative stressor is this animal model increases hypertension. Hypertension in mid-life increases a person's risk to develop later-life neurodegeneration and contributes to oxidative stress burden (24, 45, 49). Interestingly, there is evidence that anti-hypertensive drugs, such as AT1a-receptor inhibitors or hydralazine, prevent neurodegenerative processes in animal models (11, 26, 30, 44). Indeed, unpublished findings show that AT1a receptor inhibition blocked CIH-induced (AHI=10) oxidative stress generation in Sprague-Dawley single-housed male rats. Therefore, future investigations should be conducted to determine if it is androgen-oxidative stress interactions that lead to behavioral deficits or it is androgen-oxidative stress-hypertension interactions. Equivocal results have been found when using an antioxidant to block oxidative stress generation in multiple animal models (32, 38, 49), indicating inhibition of oxidative stress may not be the appropriate experimental design. However, it is possible to use hydralazine to block CIH induced hypertension, which could allow more information to be gleaned about the role of oxidative stress and hormones in the absence of hypertension.

Other future studies include investigations of female rats to determine if negative oxidative stress and androgen interactions occur in periods of androgenic physiology, such as menopause. We plan to examine this by providing female rats aromatase inhibitors, such as letrozole (0-2 mg/kg/day) in their drinking water to block the conversion of testosterone to estradiol, resulting in an androgenic state. Once we change the hormone status of the female rats, we plan to expose them to the oxidative stressor, CIH, and examine their biochemical and behavior profiles.

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