

THE EFFECTS OF CHRONIC INTERMITTENT HYPOXIA
ON OXIDATIVE STRESS AND INFLAMMATION

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THESIS

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SPECIFIC AIMS

LONG TERM GOALS:

Identify an early treatable condition which increases the risk for neurodegenerative diseases & Identify circulating biomarkers that indicate disease status and progression to aid in prevention of neurodegeneration

OBJECTIVE:

Determine the effects of CIH in the development of neurodegenerative diseases in males and establish a minimally invasive biomarker panel useful for clinical diagnosis.

HYPOTHESIS:

Mild sleep apnea contributes to increased oxidative stress which triggers an inflammatory pathway that leads to neuronal apoptosis.

AIM #1: Explore the impact hypoxia can have on increasing inflammation through oxidative stress.

AIM #2: Identify biomarkers associated with the early effects of hypoxia on oxidative stress and inflammation within brain nuclei associated with neurodegenerative diseases.

CHAPTER I

INTRODUCTION AND BACKGROUND

There is an urgent need to identify and prevent pre-existing contributors to neurodegeneration. Neurodegenerative diseases (ND) involve the significant loss of neurons within a particular region of the brain eventually leading to loss of functionality. For example, Parkinson's disease (PD) is associated with the loss of dopaminergic neurons within the substantia nigra pars compacta that leads to a loss of motor control (20) whereas Alzheimer's disease (AD) is associated with the progressive loss of neurons from the entorhinal cortex to the hippocampus to the cerebral cortex that leads to cognitive dysfunction (19). The occurrence of these diseases increases with age (87, 117) and is not typically diagnosed until the onset of a specific set of symptoms. Upon diagnosis, there are several treatment options specific to the disease which may assist in alleviating symptoms, but do nothing to promote healing or halt the progression of the disease (17, 77). Collectively, these diseases are a major source of physical, emotional and financial burden for many patients and their families, health providers, and society at large.

As the population ages (figure 1), the diagnosis of these types of diseases is expected to increase accordingly over the next 30 years. The cost of treatment of these two diseases alone is projected to exceed one trillion dollars by the year 2050 (59, 77). This is only an estimated financial cost and does not account for the toll the amount of time and the number of caregivers which will be needed over the next 35 years to help cope with the magnitude of these loss-of-

function diseases. The inability to prevent the progression of ND represents a looming health crisis. Increasingly, research is aimed at identifying early treatable events that are involved in neurodegeneration in an attempt to prevent the development of these types of diseases within a timeframe that is effective for treatment (11, 77, 137, 139, 144). The goal of this thesis is to identify a clinical pre-existing condition capable of contributing to ND characteristic hallmarks and establish biomarkers useful in early diagnosis of ND risk.

Alzheimer's Disease

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder. Symptomatically, it is initially characterized by cognitive impairment of short-term or recent memories, and eventually affects all cognitive and physiological functions, leading to death (87). Mild cognitive impairment (MCI) is a term given to early signs of cognitive impairment believed to progress toward the more severe dementia seen in AD. Pathologically, a progressive loss of neurons beginning in the entorhinal cortex which spreads to the hippocampus and frontal cortex is observed, as well as neurofibrillary tangles and beta-amyloid plaques ($A\beta$) (19, 70, 87, 91). The new PET imaging techniques can detect $A\beta$ plaques in the brain, but are not sufficient alone for diagnosis. Diagnosis is dependent on anecdotal data, a psychological evaluation using the Mini Mental State Examination (MMSE), family input, and possibly an MRI to rule out other causes of dementia (68). As of 2010, AD was the 6th leading cause of death in the United States (95). While the prevalence of AD is believed to be higher in women than men (10), studies looking at sex differences in AD have provided equivocal results in cohorts less than 78-80 years of age (91). World Health statistics report the average lifespan of women to be longer than men in most countries (5, 47, 101), so the risk of AD due to age may play a role in this observed

difference. However, a sex difference in the progression and development of the disease has been documented (10, 119), suggesting there may be different mechanisms by which men and women both progress to the same endpoint of diagnosis.

At the present rate of development, the number of new cases is expected to rise dramatically (figure 2) and the financial burden of treating dementias is projected to increase 5.6 times by the year 2050 (68). Current treatments include cholinesterase inhibitors for patients diagnosed with mild or moderate cognitive decline (MMSE score = 10-25) and memantine for more severe cases (17, 137). While there is evidence these treatments may reduce the severity of the symptoms and improve quality of life for a period of time, their efficacy is poor and they do not prevent the progression of AD (17, 137).

Parkinson's Disease

The second more prevalent neurodegenerative disease is Parkinson's disease (PD), listed as the 14th leading cause of death in 2010 (95). PD is characterized mainly by the loss of dopaminergic neurons within the substantia nigra, pars compacta, combined with the presence of α -synuclein Lewy bodies within affected neuronal cell bodies (20, 117). Symptomatically, patients exhibit tremors and bradykinesia associated with the loss of motor neuron control (20, 27). Age is highly implicated in the development of this disease as well as genetic factors. Men are more than twice as likely to develop PD as women (46, 126), illustrating yet another sex difference contributing to the development of ND. Similar to AD, the current cost of treatment for PD in the United States is in the billions of dollars and is expected to more than double by 2050 (figure 3) (77). Additionally, current treatment with levodopa to increase dopamine

signaling temporarily relieves symptoms, but is not capable of preventing the progression of further neuronal loss (25).

Due to the lack of effective pharmaceutical treatments, current methods of diagnosis do not appear to occur within a positive therapeutic window. Definitive diagnoses of ND depend upon post-mortem autopsies to identify the characteristic brain morphology of each disease (19, 20). Doctors are reliant upon psychological examinations, anecdotal data, and the perceived progression of characteristic symptoms to make their diagnoses. Often the initial symptoms are too subtle to catch early on (25). Many efforts are directed towards developing definitive early diagnostic tools such as blood marker tests or brain scans (24, 43, 119, 153).

The insufficiency of effective treatments to prevent progression or promote healing from ND suggests that when a patient presents with symptoms, neurodegeneration has progressed beyond a point of no return. Therefore, it would be beneficial to identify early physiological events capable of contributing to neurodegeneration in an attempt to prevent development. In that vein of thought, factors that are common to ND may provide useful markers for research to identify points that may be treatable to prevent neurodegeneration. Two particular hallmarks of ND are worth further investigation: inflammation and oxidative stress.

Inflammation

One of the hallmarks of ND is elevated inflammation within the specific regions affected. Whether this characteristic originally develops as a result of neuronal insult or is a major contributor to initial neurodegeneration remains to be elucidated. What is documented is pathological levels of cytokines within post-mortem tissue and CSF of patients who have suffered from ND as well as the capability of these cytokines to trigger processes leading to additional neuronal apoptosis (7, 23, 42, 94, 116). This evidence is worthy of further investigation.

Immune System Function

The vertebrate immune system plays a vital role in protecting against invading pathogens and wound healing. Leukocytes perform immune surveillance and, upon encountering specific molecular signals, mobilize cellular resources to efficiently quarantine and remove potentially toxic insults (39). Some of those signals are soluble proteins collectively known as cytokines (114). Cytokines are released by cells in response to a perceived injury and serve to attract immune cells and initiate inflammation that leads to differentiation into adaptive profiles specific to the insult. Overall, the immune system performs a crucial role in maintaining homeostasis.

While the acute immune response is beneficial, chronic inflammation is implicated in many metabolic and auto-immune disorders (63, 82). Untreated and/or under-treated infections or repeated injury to tissue elicits overexpressed cytokines. This leads to excessive chronic recruitment of lymphocytes that damages tissue rather than protecting it.

Cytokine Profiles

Cytokine expression involves both pro-inflammatory and anti-inflammatory elements which act synergistically in healthy tissue to promote effective healing. For purposes of this thesis, “pro-inflammatory” will refer to cytokines whose role is to recruit innate immune cells which non-specifically phagocytose cells they are attracted to (82, 90). Activated granular immune cells are crucial in the initial response to contain pathogens and reduce the exposure of healthy surrounding tissue to the insult (39). For example, the classic microglial role, designated M1, is removal of damaged tissue (26). The Th1 cells are helpful in providing the first defenses against an invading bacterial or viral infection (115, 118). Because they can inadvertently damage the same tissue they are recruited to if not regulated or if chronically present, these cells are classically termed “pro-inflammatory.” “Anti-inflammatory” cytokines will refer to molecules which attenuate pro-inflammatory responses by either triggering pathways that attenuate pro-inflammatory ones or play a role in differentiating naïve lymphocytes into specific adaptive or protective profiles. Adaptive immune cells, such as Th2 cells, show specificity for pathogens and will only degrade those cells with markers of the pathogen they are directed to, as well as release cytokines that block the innate response to promote cell viability (115, 118). The M2 profile of microglia is believed to be neuro-protective by increasing neuronal genesis and differentiation (26). Which profile these lymphocytes express is determined by the cytokine proportion in the local environment. Pro-inflammatory cytokines induce apoptotic pathways. Anti-inflammatory cytokines induce protective effects. Typically, these exist in a balance that promotes homeostasis (table 1).

Chronic elicitation of cytokines can tip the scales from homeostatic processes toward a pro-inflammatory profile, ultimately leading to auto-immune disorders (figure 4). Foremost

among cytokines is interleukin-1 (IL-1). IL-1 was the first cytokine to be reported and has two isoforms, IL-1 α and IL-1 β (56, 57, 129). IL-1 α is constitutively expressed, but IL-1 β must be cleaved from its pro-form by caspase-1 (129). Caspase-1 is expressed in response to injury or infection (82, 129), making IL-1 β a crucial initial signal in the recruitment of leukocytes. IL-1 β is capable of both autocrine and paracrine signaling to propagate both itself and other cytokines (82).

This ability to self-perpetuate the IL-1 β signal is a focal point in inflammatory disorders. Left unchecked, IL-1 β will continuously recruit phagocytic leukocytes and promote a pro-inflammatory profile (figure 4). In acute responses, this is necessary and protective to the organism. IL-1 β deficient models do not survive infection or injury, partly because of the role IL-1 β plays in recruitment (93). It is the chronic activation that has been correlated to autoimmune and metabolic diseases (63, 134) suggesting care should be taken to avoid scenarios leading to prodigious IL-1 β activation.

Along with IL-1 β , tumor necrosis factor – α (TNF- α) is another crucial player in the immune system. TNF- α is a pyrogenic capable of activating sickness behavior and fever through the hypothalamic-pituitary-adrenal (HPA) axis that aids in fighting off pathogens (40, 146). As its name suggests, it also plays a role in preventing tumorigenesis (110). When overexpressed, TNF- α is responsible for cases of systemic shock leading to death (figure 4). Therefore, production of TNF- α is typically highly regulated.

Interleukin-6 (IL-6) is a third partner in prototypical inflammation. It is released by monocytes in response to IL-1 β or TNF- α and is responsible for altering gene expression in somatic cells which leads to apoptosis (16, 114), the programmed cell death of an injured cell

(figure 4). This allows for controlled clearance of pathogens or damaged cells while minimizing risk to surrounding tissues.

As can be seen, these three cytokines, while considered to be “pro-inflammatory,” play beneficial roles in protecting the homeostatic environment and health of an individual. It is not until they are chronically overexpressed that detrimental effects of pro-inflammatory conditions occur.

Inflammation as a Hallmark of Neurodegenerative Diseases

It was with medical attempts to transplant tissues that the role of the immune system within the central nervous system began to be defined. It was observed in the early 1900s that tumors or organs would proliferate longer when transplanted into certain areas of the body rather than others (8). The idea of immune-privileged sites within the body began to proliferate. These sites are believed to be protected from the effects of the immune system through a variety of mechanisms, preventing host tissue rejection of the transplant (8). Classically, the brain is one of these sites. The survival of implanted tissues that did not disturb the ventricles and the lack of observable lymphatic drainage vessels, as well as the deficit of measurable inflammatory markers within the brain under scenarios with elevated peripheral inflammation, perpetuated this idea (8, 89). Indeed, the existence of the blood brain barrier (BBB), which utilizes tight junctions and limited transporters, contributes to this status.

In spite of this immunological privilege, as assay methods have become more sophisticated, cytokines have been measured within brain tissue under both healthy and diseased conditions (16, 82, 129, 143, 154) and evidence of lymphatic vessels has recently been

discovered (81). Increasingly, cytokines have been shown to play a role in behavior, cognition, neuroinflammation, and as neuromodulators (76, 92, 143).

An increase in both IL-1 β and TNF- α has been documented in cases of Alzheimer's disease and Parkinson's disease (16, 23, 129). IL-1 β and IL-6 levels are significantly increased in the CSF of patients diagnosed with Alzheimer's and Parkinson's disease (16). Substantial elevation of IL-1 β has also been observed in post-mortem brain tissue of Alzheimer's disease patients as well as those with MCI (56, 57). Similarly, increases of IL-1 and IL-6 within the substantia nigra have been observed in patients with Parkinson's disease (116). These pro-inflammatory markers are capable of triggering apoptotic pathways and recruiting macrophages that can lead to further neuronal cell death, creating a cycle that is self-perpetuating, leading to extensive neurodegeneration.

IL-1 β is also associated with altered protein expression patterns similar to those observed in ND. Cyclooxygenase-2 (COX2) is one protein expressed following reception of the IL-1 β signal. COX2 expression is implicated with an increase in α -synuclein deposition (123). α -synuclein is a primary protein comprising Lewy bodies, one of the pathological features of Parkinson's disease (20) and accumulating in some cases of Alzheimer's disease (123). IL-1 β has also been associated with elevation of inducible nitric oxide synthase (iNOS) transcription and signaling, which is implicated in apoptosis (133). Chronic elicitation of these proteins over a lifetime of neuronal insult could be the catalyst that tips the homeostatic balance toward neurodegeneration rather than neurogenesis and proliferation.

Potential Sources of Inflammation

A major source of cytokines within the CNS is microglia. However, IL-1 β and TNF- α have been documented to be released by neurons (table 1) as well (129). The conditions under which each cell type releases cytokines appears to be dependent on the environmental stress it is experiencing and the brain region the stress occurs in. For example, systemic LPS stimulation generally leads to IL-1 β release from microglia around the choroid plexus of the circumventricular organs which is accessible by systemic blood flow (146). In contrast, lesions and increased glutaminergic stimulation to hippocampal neurons leads to a temporal and spatial pattern of IL-1 β expression by neurons themselves days before the signal is observed in microglial residents of those regions (138, 143). Additionally, evidence of inflammation as represented by an elevation of COX2 expression has been documented in neuronal cell lines devoid of glial populations (138, 143). This suggests that neurons themselves are capable of mobilizing an immune response when they have been taxed beyond their own resources.

The precursor to IL-1 β , pro-IL-1 β , is stored within the cell and then cleaved to IL-1 β by caspase-1 and released upon a perceived insult to the cell, activating lymphocytes and vascular endothelium to induce an inflammatory response that should promote healing under homeostatic conditions. These inflammatory markers are capable of triggering apoptotic pathways and recruiting macrophages that can contribute to further neuronal cell death, creating a cycle that is self-perpetuating. Identifying physiological situations that lead to neuronal cytokine production and pro-inflammatory imbalance would be helpful in promoting preventative measures capable of restoring homeostasis and preventing neurodegeneration.

Oxidative Stress

Another hallmark of neurodegenerative diseases is oxidative stress (30, 67). Oxidative stress is an imbalance in the cell's ability to manage reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in accumulation of reactive molecules. This can be due to a lack of enzymes which neutralize ROS/RNS or an overproduction and accumulation of reactive species beyond the cell's capabilities to neutralize them. These highly reactive molecules can modify other proteins within the cell, leading to a loss or change of function by those proteins. This creates an environment of damage that triggers caspase-1 to cleave pro-IL-1 β , releasing IL-1 β (93), and leading to an elevation of inflammation (figure 5).

Sources of ROS and RNS

Reactive oxygen species, such as hydroxyl radical (OH \cdot), hydrogen peroxide (H₂O₂), peroxynitrite (OONO \cdot) and super oxide anion (O $_2^{\cdot-}$), exist within cells at basal physiological conditions. These molecules play an integral role in energy production, neuronal signaling, cell proliferation, and immune responses (31, 84, 128, 149).

Typically, due to their highly reactive nature, their existence is tightly regulated and scarce within the cell. Most ROS are within compartments such as mitochondria or peroxisomes where they can safely perform their function or be stored for usage without coming into contact with other cellular components (93, 128).

Endogenously, O $_2^{\cdot-}$ is generated within mitochondria during normal energy metabolism by nicotinamide adenine-dinucleotide phosphate oxidase (NOX), Xanthine oxidase, and uncoupled nitric oxide synthase (NOS) (93, 128). It is converted to H₂O₂ by superoxide

dismutases (SOD) which is further reduced by peroxidases or catalase depending on the concentration and location of H_2O_2 .

Nitric Oxide (NO) is a reactive nitrogen species RNS synthesized by NOS isoforms and acts as second messenger in several pathways to regulate blood pressure, provide immune defense by phagocytic cells, and serve as a neurotransmitter (113, 141, 146). It is a highly transient molecule with a half-life of only a few seconds, making it difficult to measure in vivo. In neurons, it is generated by neuronal nitric oxide synthase (nNOS). However, under hypoxic conditions these enzymes become uncoupled, producing $O_2^{\cdot-}$, and inducible NOS (iNOS) is transcribed (79, 141). iNOS produces NO in much greater concentrations than nNOS. When OH^{\cdot} and NO coexist abundantly, they interact to form the highly reactive $OONO^{\cdot}$ (141). $OONO^{\cdot}$ reacts with proteins and irreversibly modifies tyrosine residues on proteins in a process called nitrosylation (22, 79). Nitrotyrosine (NT) can be measured as an indicator of NO presence.

Physiological Function of ROS and RNS

Collectively, ROS/RNS drive mechanistic processes necessary for proper cell function at homeostasis. Within neuronal and endothelial cells, ROS are elevated in response to hypoxic conditions and upregulate the transcription of hypoxia inducible factor (HIF) (31, 128). HIF has 2 isoforms, HIF-1 and HIF-2. HIF-1 α is normally degraded, but is implicated in the hypoxic response. It binds the HIF response element (HRE) and localizes to the nucleus to initiate transcription of vascular endothelial growth factor (VEG-F), erythropoietin (EPO), and glycolytic enzymes (128). As a whole, these enzymes coordinate increased blood flow to deliver more O_2 to the tissue while simultaneously dampening the need for O_2 for energy production.

ROS play an integral part in immune responses as well. The activation state of immune cells is related to the concentration of ROS within their mitochondria (141). They also exist in peroxisomes which are released by phagocytic cells in oxidative bursts to kill invading cells and degrade pathogenic material (93). The ability to elevate HIF-1 α regulated pathways also contributes to IL-1 β signaling and its associated inflammatory pathways.

RNS similarly play an integral role in both basal and adaptive cellular responses. NO in endothelial cells maintains vascular tone and blood pressure (84). In neuronal cells, it is a vital second messenger and neurotransmitter, able to diffuse across membranes rapidly and initiate signaling pathways (141). Under hypoxic conditions, iNOS produces NO in large quantities (133), which most likely plays a role along with ROS to rapidly restore O₂ concentrations to normal by increasing blood flow and triggering neuronal pathways to restore breathing and decrease energy usage (128).

ROS are buffered by antioxidant systems such as glutathione peroxidase, peroxiredoxins, and catalase (141). When the amount of ROS produced overwhelms the capacity of these buffering systems, OS occurs. OS oxidizes lipids, DNA, and proteins (141). This oxidized environment leads to cellular and tissue damage.

Because ROS/RNS are so transient, their existence is difficult to monitor. Instead, downstream indicators of their actions must be assayed to quantify their presence. Products of lipid peroxidation are the formation of malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal (HNE). 8-hydroxy-2-deoxyguanosine (8-OHdG) is an indicator of DNA damage due to ROS. OS leads to the formation of carbonyls on proteins by ROS and advanced glycation end products (AGEs) or advanced oxidative protein products (AOPP) in addition to nitrotyrosine (NT) by

RNS (9, 141). Assays which quantify the levels of these types of end products as well as antioxidant enzyme concentrations are helpful in elucidating the role OS plays in pathology.

Oxidative Stress as a Hallmark for Neurodegeneration

The existence of these OS byproducts has been well documented in cases of neurodegeneration. In cases of AD, elevated HNE levels and protein carbonylation, AGE, MDA, and NT within postmortem brain tissue (9, 21, 141) have been observed. Additionally, A β , a key component of the senile plaques associated with development of AD, is upregulated under OS conditions (31). A β can be transported into mitochondria where it interferes with the electron transport chain, further elevating OS (31). This environment could conceivably contribute to exacerbating the pathological condition of neurons that leads to dementia seen in AD.

Depletion of dopaminergic cells in the substantia nigra (SN) is observed in patients diagnosed with PD and is believed to be a major contributor towards the observed symptoms. The metabolism of dopamine is a highly oxidative process, so these cells exist under high levels of OS at basal conditions. Regardless, elevations of 8-OHDG have been documented in the brain tissue of patients with PD (9). Additionally, significant depletion of the glutathione buffer system in the SN has been implicated as an early event in the progression of PD (141). Another characteristic of the disease is the formation of Lewy bodies composed of α -synuclein. α -synuclein is involved in maintenance of the mitochondrial membrane and accumulation of this protein leads to mitochondrial dysfunction that can further elevate OS conditions (31). These factors all suggest a fundamental role for OS in the pathology of PD.

Oxidative Stress as a Function of Age and Sex

The free radical theory of aging proposes that OS contributes to the accumulation of damaged DNA and proteins over time, resulting in cellular loss and aging of an organism. There are many studies that corroborate this theory since it was first proposed in the 1950's (141). Studies show OS is naturally elevated as aging occurs (30, 31, 141) (figure 6). This elevated OS is associated with many metabolic age related diseases (9, 65, 67, 79, 93, 128). There appears to be an age-associated decline in oxidative phosphorylation, leading to elevated ROS production as well as a decrease in the ability of cells to buffer ROS (31). This elevated cellular ROS contributes to the overall OS load as aging occurs.

Specific age related damage appears to occur most extensively in mitochondrial DNA (mDNA) (31). Because 1-3% electrons leak across the electron transport chain (31, 128), they interact with molecular O₂ to form superoxide anion, causing mDNA deletions. mDNA deletions accrue over a lifetime, leading to respiratory failure and eventual death of the cell (31). Typically, damaged mitochondria are removed through mitophagy (58, 98). An over accumulation of damaged mitochondria can lead to dysfunctional mitophagy processes and contribute to elevated ROS (93, 128).

Interestingly, OS appears to accumulate more rapidly in men than women as they age (figure 6) (30, 48). In vitro studies of striatal neurons and astrocytes from both genders have shown those derived from males to be more susceptible to OS than female cells are, even though the levels of ROS buffering systems were not different (48). This was correlated with a sex difference in a mitochondrial protein expression in which female cells exhibited more mitochondria than male cells. The combined age related increase in oxidative stress and the higher susceptibility to OS by men could contribute to a higher risk of neurodegeneration for

males. Additional research into mechanisms which influence OS elevation in a sex specific way could provide valuable insight into pre-existing conditions which lead to neuronal loss.

Sleep Apnea

A common comorbidity of neurodegenerative diseases is obstructive sleep apnea (OSA) (4, 34, 45, 50, 148). Sleep apnea may prove to be a major contributor to the risk of neurodegeneration in men. Existing prior to neurodegeneration, it shares many hallmarks of ND and is more prevalent in men than women (41, 88, 107, 112, 150). These factors make OSA a potential modifiable early risk physiological condition and treatment of OSA may prevent development of ND.

Characteristics of Sleep Apnea

Obstructive sleep apnea is characterized by repeated occurrences of partial or full airway obstruction during sleep known as hypopneas or apneas respectively (38). This causes a repetitive decrease in O₂ availability during sleep on a nightly basis, as well as hypercapnia. These conditions lead to episodes of wakefulness and physiological changes such as hypertension and altered intrathoracic pressure.

Diagnosis of OSA is dependent on completion of a sleep study (122, 150), during which a patient's O₂ saturation, the number of arousals, and airflow is monitored. An apnea is classified as a complete obstruction of airflow or pressure. A hypopnea is recorded when a reduction in airflow $\geq 30\%$ for 10 seconds or longer is accompanied by O₂ desaturation $\geq 4\%$. Together, the number of times either of these events occur per hour makes up the apnea/hypopnea index (AHI). A patient with an AHI of 5-15 events/hour is considered to have mild sleep apnea according to current American Academy of Sleep Medicine guidelines (122). Moderate sleep apnea is designated for AHI in the range of 15-30 events/hour, while an AHI more than 30 is severe.

Treatments of sleep apnea involve methods to prevent obstruction. They may include wearing a mouth guard while sleeping which prevents mechanical obstruction or a weight loss program. Severe cases are prescribed continuous positive airway pressure (CPAP) which involves sleeping with a face mask and mechanical application of positive pressure to maintain an open airway (86).

Prevalence of Sleep Apnea in the Population

OSA impacts about 5% of the population. However, since many people do not realize they have difficulty breathing while sleeping or may not chose to undergo a sleep study, there are potentially more individuals with OSA than are currently diagnosed. The frequency of occurrence is estimated to be much higher than the results by polysomnography report and has risen over the past 20 years. This is supported by most clinical cohorts showing a much higher incidence of OSA than what is reported in the general population (41, 107, 112, 150).

The incidence of OSA is positively correlated with body mass index (BMI) and age in men and women. The first observations of sleep apnea were based on daytime sleepiness and obstructed nighttime breathing in obese patients and BMI remains the largest contributor (38). Men are 2-3 times more likely to develop OSA and experience more severe symptoms than women (41, 107, 150, 151). In one study, 13% of men were found to have OSA as compared to 6% of women (107) and the men/women odds ratio (OR) for OSA was found to be 1.2 for $AHI \geq 5$ and 3.0 for $AHI \geq 30$ in a study by Duran, et al. (41). Age as well has been found to increase both the incidence and severity of OSA. In individuals 25 – 60 years, the rate of occurrence was up to 5 times greater as aging occurred. The Cleveland Family Study and other studies validate this finding (38, 41, 107, 112, 150). The significant correlation with age as well as the higher

incidence in men makes OSA a potential candidate as an early contributor to elevated risk of ND in men.

Sleep Apnea as a Comorbidity of Neurodegenerative Diseases

Indeed, OSA has been documented as a comorbidity of neurodegeneration. Sleep disordered breathing has been associated with earlier cognitive decline in AD patients (73, 102). Additionally, 40% of men with AD in the Texas Alzheimer's Research Care & Consortium Cohort (TARCC) report experiencing sleep disturbances as opposed to 7.5% of men who are cognitively intact in the cohort (figure 7) (Cunningham, unpublished data). Additionally, 80-90% of men diagnosed with PD also are diagnosed with OSA (4, 125). The changes in cerebral blood flow and cortical activity leading to altered molecular responses observed in patients with OSA is believed to contribute to a negative impact on neuronal function and degeneration (12, 45, 105, 120). The severity of OSA has been positively correlated to impairment in executive function, episodic memory, and motor skill deficits (45, 78). Two core characteristics of AD, tau phosphorylation and amyloidogenesis, have also been positively correlated to the presence of OSA (120).

Treatment of OSA with CPAP for at least four hours every night has shown promising results in some areas of cognitive dysfunction (45). Experiments by Ancoli-Israel, et al. showed mild cognitive improvement in patients with comorbid mild/moderate OSA and AD after only 3 weeks of compliant treatment (3, 45). Following this, a cohort of patients with severe OSA and mild-moderate AD, as indicated by MMSE score ≥ 15 , was monitored for an average of 3.3 years. Those demonstrating good compliance of the CPAP protocol for 12-24 months did not have a significant decline in MMSE scores as opposed to non-CPAP users who showed a

significant worsening of cognitive abilities over the same time frame (139). Additional studies have demonstrated improvements in executive function and episodic memory following treatment of OSA, although equivocal results have been found in those targeting manual dexterity (45). These studies are highly indicative of a causal relationship between OSA and neuronal dysfunction which may be reversible. Future studies elucidating the early contributions OSA may make toward neurodegeneration would provide critical evidence helpful in developing preventative treatments.

Sleep Apnea Association with Inflammation and Oxidative Stress

In both clinical and rodent models of OSA, elevated inflammation is a characteristic hallmark. IL-6 and IL-10 are systemically elevated in children with OSA and return to baseline upon adenotonsillectomy to reduce OSA (55, 71). In adult patients with OSA, systemic TNF- α , IL-8, and IL-6 have all been documented to be elevated (86). Treatment of OSA has provided mixed results in the normalization of these cytokines. The intermittent hypoxia (IH) associated with OSA is correlated with elevated microglial response in the CNS as well as inflammation within the carotid bodies which modulate the central response to IH (133). This altered inflammatory profile may accumulate over a lifetime of non-treatment, is indicative of inflammation observed in neurodegeneration, and may be a driving causal force of ND.

The intermittent hypoxia (IH) experienced by OSA patients is similar to repeated bouts of ischemia and reperfusion that are responsible for elevated oxidative stress in other metabolic diseases. This implies IH is capable of similarly elevating OS in OSA. Indeed, circulating OS has been documented in clinical and animal models of OSA (79, 103, 127, 147).

Evidence points to this elevation in OS being a catalyst for consequential inflammation and eventual neurodegeneration. In animal models of IH, NOX activity has been associated with the cognitive effects of IH and knockout of NOX or administration of erythropoietin (EPO) or growth hormone (GH) attenuates the negative cognitive impairments in IH by modulating damage due to oxidative stress (35, 80). Elevations of NOX activating subunits have been observed in animals undergoing IH (35), suggesting it is indeed a major contributor to neurodegeneration due to elevated OS caused by IH.

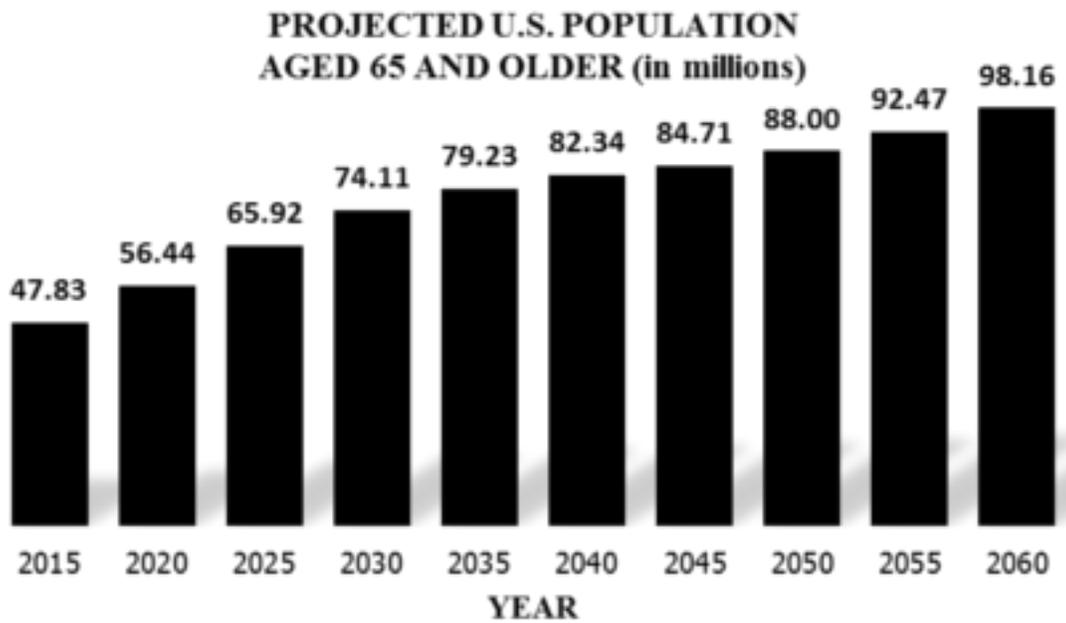
Another factor to consider in the generation of ROS by OSA is angiotensin II (ANG II). Circulating ANG II is elevated in OSA and plays a major role in the cardiovascular response (44). It has also been shown to elevate ROS generation by stimulation of NOX2 and other pathways (49, 155). ANG II is also associated with increased blood pressure in OSA, which may be another contributor to OS (124). With the incessant nightly elevation of ANG II over a lifetime of OSA, the rate of ROS accumulation and consequential downstream signaling effects as well as oxidation of cellular components could be greatly accelerated. The effects of ANG II in OSA also vary by sex (151), as seen with OS, the incidence of OSA, and the development and progression of ND. The cumulative action of all these factors deserves further investigation into treatment of OSA as a preventative therapeutic to prevent neurodegeneration.

To explore the early impact OSA can have on OS and inflammation, a model of chronic intermittent hypoxia (CIH) was used. CIH in rodents mimics the hypoxemia experienced by OSA patients. Rodents are housed for a period of time in chambers with adjustable air supplies. During the sleep phase, oxygen concentrations are periodically manipulated to recreate the hypopneas characteristic of OSA. For this experiment, oxygen was reduced over a period of 90 seconds to a concentration of 10%, held at 10% for 90 seconds, and then returned to the normal

concentration of 21% over 90 seconds. 21% O₂ was maintained an additional 90 seconds before the cycle repeated. This occurred over a period of 8 hours, reflecting an AHI of 10, which is considered clinically mild sleep apnea. This model allows for characterization of the impact repeated hypoxic events while sleeping has on pathology. Following seven days of CIH, rats were deeply anesthetized, and plasma and brain tissue punches collected for assessment.

FIGURE 1

PROJECTED U.S. POPULATION AGED 65+, 2015-2050



Source: U.S. Census Bureau, Population Division
Release Date: December 2014

Figure 1 Projected U.S. population aged 65 and older to the year 2060. The rise in the elderly population elevates the prevalence projections of age-related diseases. (145)

FIGURE 2

PROJECTED RISE IN ALZHEIMER'S DISEASE, 2010-2050

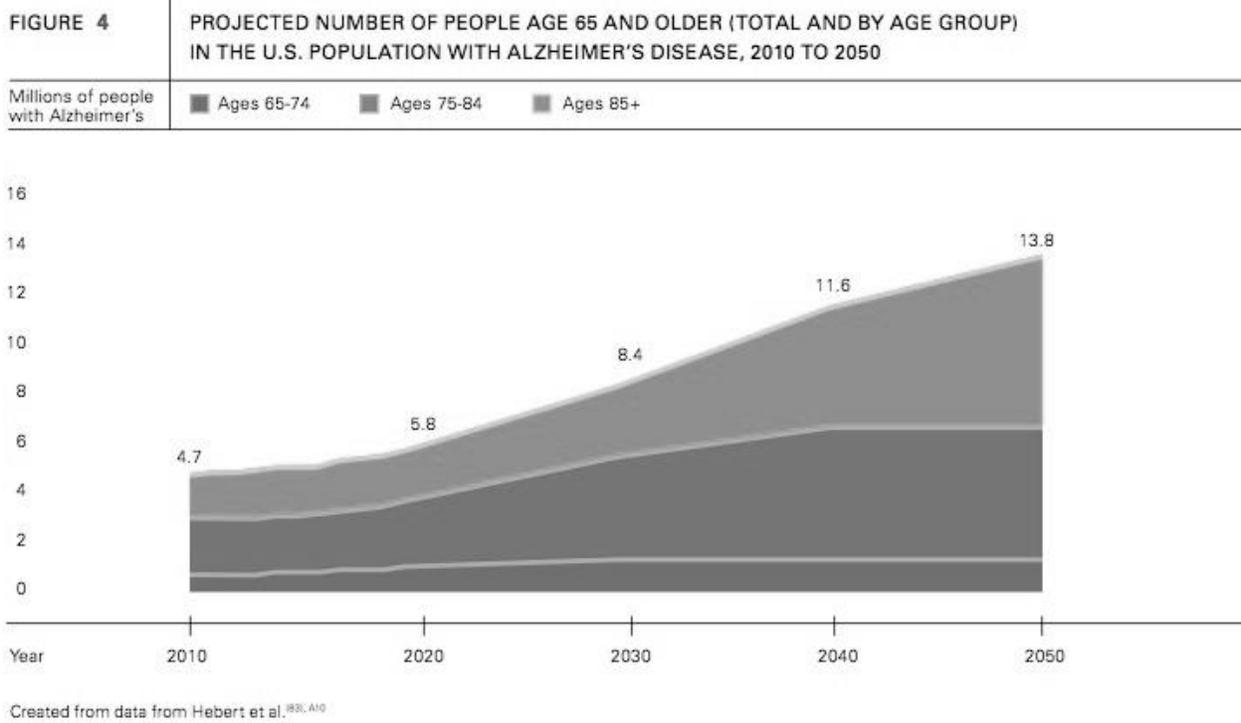


Figure 2 The number of people projected to develop Alzheimer's disease is expected to rise dramatically by the year 2050. (68)

FIGURE 3

MEDICAL COSTS OF PARKINSON'S DISEASE OVER TIME

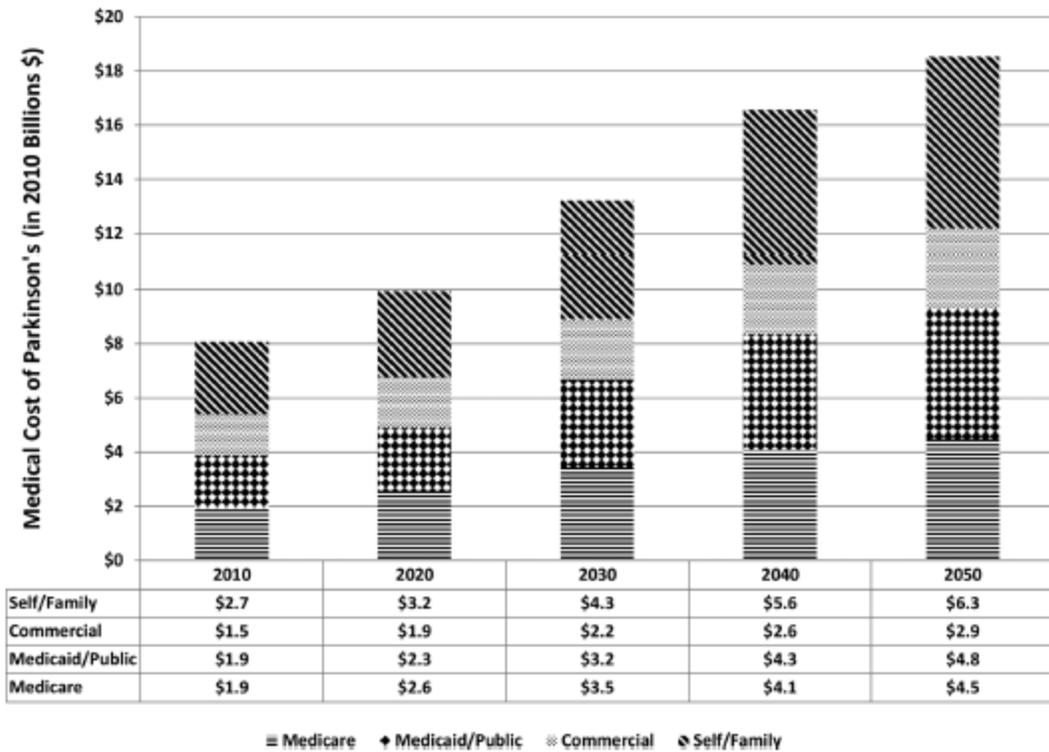


Figure 3 The financial burden associated with the treatment of Parkinson's disease will more than double by the year 2050. (77)

FIGURE 4

INFLAMMATORY CYTOKINE CYCLE

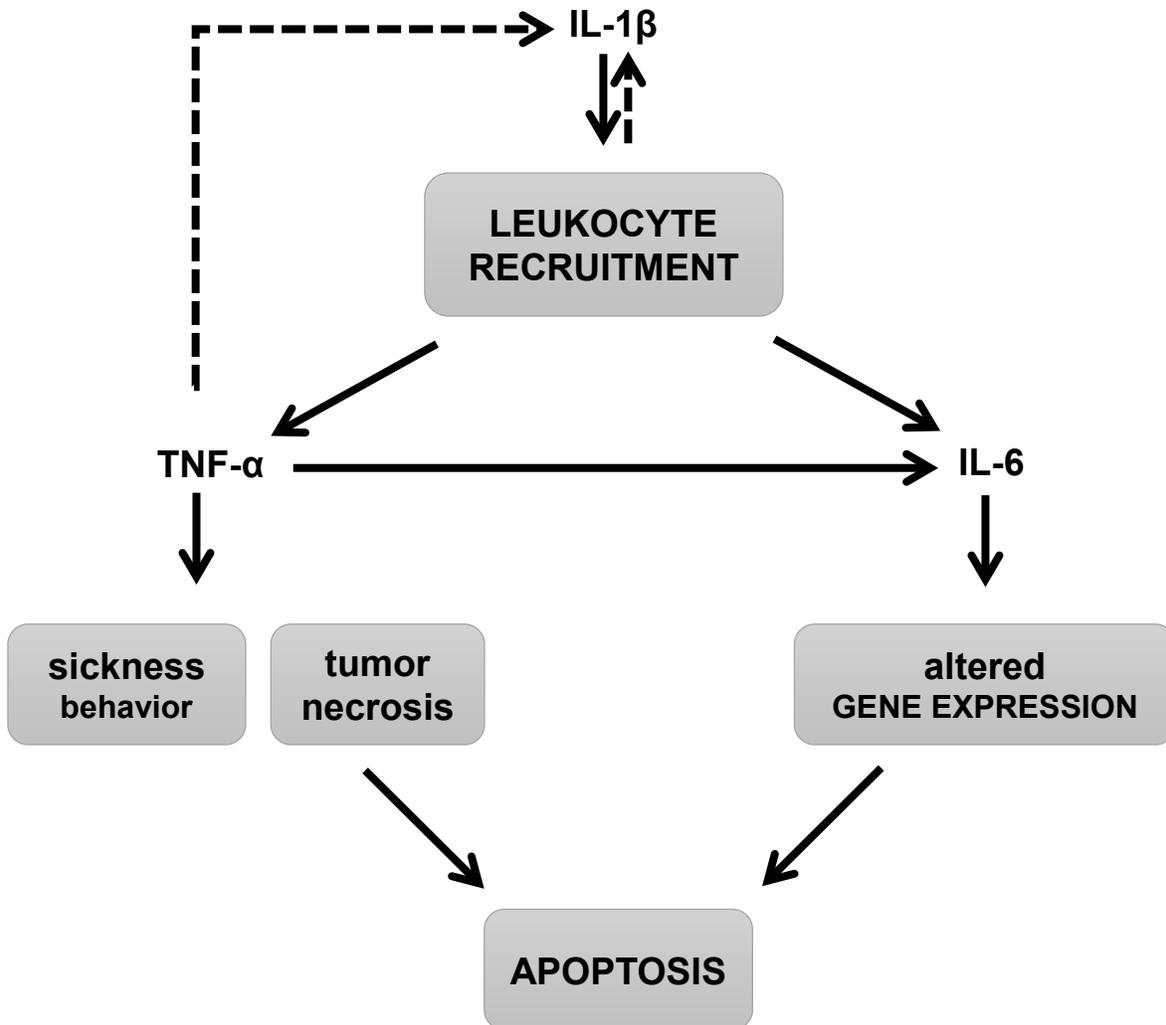


Figure 4 IL-1 β , TNF- α , and IL-6 contribute to inflammatory processes necessary to maintain homeostasis.

FIGURE 5

OXIDATIVE STRESS CONTRIBUTES TO INFLAMMATION

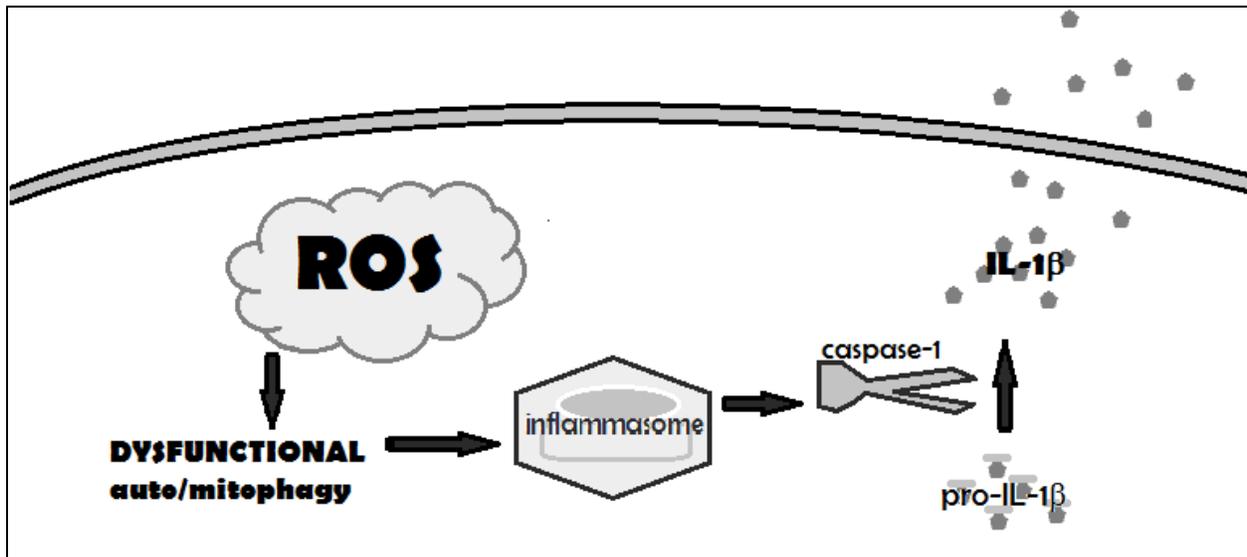


Figure 5 Reactive oxygen species in lead to dysfunctional cellular processes which lead to inflammasome assembly. The inflammasome activates caspase-1 to cleave pro-interleukin-1 β to the active interleukin-1 β , triggering inflammatory signaling.

FIGURE 6

OXIDATIVE STRESS LEVELS BY AGE AND GENDER

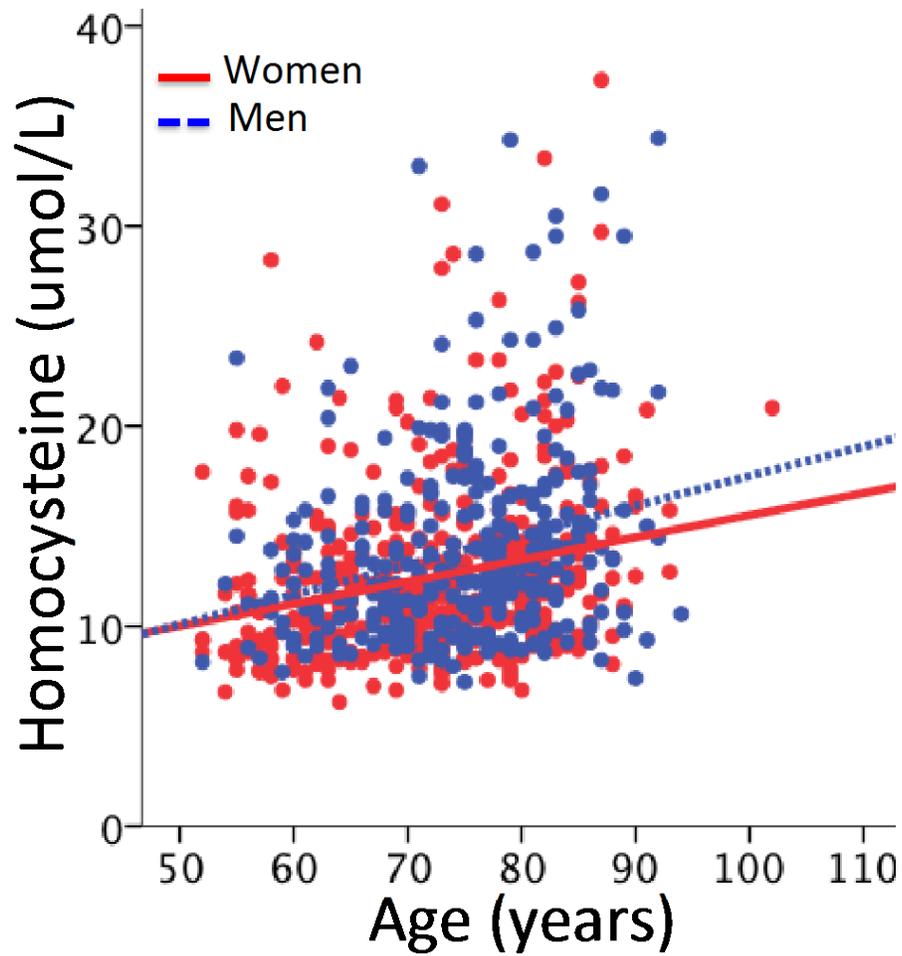


Figure 6 Oxidative stress, as measured by homocysteine, significantly increased with age in men ($r^2=0.006$, $n=320$) and women ($r^2=0.057$, $n=454$). However, men have significantly higher OS levels than women ($p<0.001$). Texas Alzheimer’s Research Care & Consortium cohort (TARCC)

FIGURE 7

PERCENT MALES WITH SLEEP DISTURBANCES

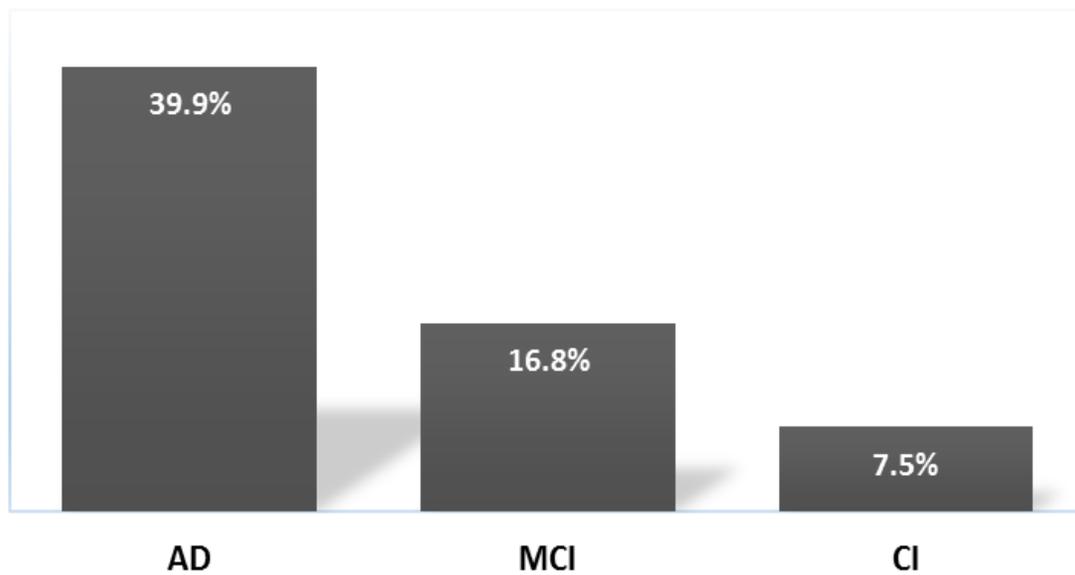


Figure 7 Caucasian males diagnosed with AD are over 5x more likely to experience sleep disturbances than men who are cognitively intact. Texas Alzheimer’s Research Care & Consortium cohort (TARCC)

TABLE 1

CYTOKINES OF INTEREST

cytokine	microglia	neuron	pro-	anti-
<i>IL-10</i>	+	-	+	+
<i>IL-13</i>	+	-	-	+
<i>IL-4</i>	+	-	-	+
<i>IL-1β</i>	+	+	+	-
<i>IL-6</i>	+	-	+	-
<i>TNF-α</i>	+	+	+	-
<i>KC-Gro</i>	+	-	+	-
<i>IFN-γ</i>	+	-	+	+
<i>IL-5</i>	+	-	+	+
<i>IL-2</i>	+	-	+	+

Table 1 Cytokines of interest for this study and known role in neuroinflammation (82, 90, 99, 114, 118, 131, 154)

CHAPTER II

MILD CHRONIC INTERMITTENT HYPOXIA INDUCES OXIDATIVE STRESS AND INFLAMMATION IN MALE RATS

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Abstract

Obstructive sleep apnea (OSA) is a common comorbidity of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases and stroke. It occurs more frequently in men than women. The risk of developing OSA increases with age and body mass index. There is evidence OSA contributes to an inflammatory profile that is similar to inflammation associated with neuronal degradation. Both clinical and animal models of OSA exhibit elevated oxidative stress. Interestingly, men have higher oxidative stress levels than women. It is likely OSA elevates oxidative stress, leading to chronic inflammation that can increase the risk of neurodegeneration. Circulating angiotensin II is capable of increasing oxidative stress. This may be one mechanism by which oxidative stress is elevated in OSA. Biomarkers, such as oxidative stress and inflammatory markers in plasma may indicate risk level. To investigate this, we subjected male rats to seven days of mild chronic intermittent hypoxia (AHI = 10) then assessed oxidative stress and cytokine levels in plasma and brain regions implicated in the progression of neuronal diseases. Additionally, angiotensin II receptors in the hypothalamus were knocked out by adenovirus injection in a subset of animals. CIH significantly elevated oxidative stress and inflammation and blocking AT1a receptors abrogated the observed increase. Regional differences in oxidative stress and inflammation in the brain were also observed, and these differences are associated with plasma oxidative stress levels.

The occurrence of obstructive sleep apnea (OSA) is increasing worldwide (83) and is a common comorbidity associated with neurodegenerative diseases such as stroke, Alzheimer's disease (AD), and Parkinson's disease (PD) (4, 12, 45, 78, 139, 148). It occurs 2-3 times more frequently in men than women, and the prevalence of OSA increases with age (41, 107, 112, 150, 151). A key characteristic of OSA is alternating periods of reduced oxygen inspiration (apnea or hypopnea) while a patient sleeps that leads to a chronic hypoxic environment. The severity of OSA is often determined by the apnea/hypopnea index (AHI), which measures the number of times per hour a hypoxic event occurs during sleep.

There is evidence that hypoxic events can be either neuroprotective or neurotoxic depending on the severity, frequency, and duration of the hypoxia (13, 18, 53, 54, 69, 72, 79, 96, 97, 104, 105, 120, 133, 136, 147). Acute hypoxic events appear to upregulate protective pathways within neurons (79, 104, 132, 136). In contrast, chronic intermittent hypoxia (CIH), which occurs repeatedly during sleep every night, has been associated with increased neurodegeneration (51, 54, 72, 79, 96, 97, 105, 120, 121, 133, 147). CIH exerts its effects in a variety of brain nuclei, which can impact different functions such as cognition, motor ability, and homeostatic functions (2, 28, 53, 105, 147). There is evidence that reduction of the hypoxemia and inhibition of molecular pathways triggered by CIH can reduce the risk of neurodegeneration (35, 52, 53, 75, 80, 139).

The rodent model of CIH allows for the study of hypoxia-reoxygenation mechanisms, similar to those experienced by patients with sleep apnea that could lead to tissue damage in many comorbid diseases. This model has been well characterized to mimic the hypoxemia experienced by sleep apnea patients (51). For this study, we used a model of mild hypoxemia (AHI = 10) (38, 122) for 7 days to investigate the contribution early exposure to CIH has. Many

studies have looked at the impact of severe hypoxemia (AHI>30). Longer CIH protocols have been shown to produce region specific OS dependent apoptosis in the central nervous system that can be associated with cognitive deficits (97, 133, 147) Further, these effects are exacerbated in aged animals (54). However, few studies have examined the early impact of mild to moderate hypoxia experienced by many patients, much less its effects on the inflammatory system within different regions of the brain.

To determine how CIH can contribute to inflammation in different brain regions, we selected five nuclei representative of different functions within the major regions of the brain. Nuclei from the brain stem involved in maintenance of homeostatic functions were the rostral ventrolateral medulla (RVLM) and the solitary tract nucleus (NTS). These nuclei have been implicated in the hypertension associated with CIH (1, 28) and are believed to be affected by circulating inflammation (37, 146). A midbrain nucleus, the substantia nigra, consists of dopaminergic neurons and is involved in reward and movement (27, 61). Loss of dopamine neurons in this region is implicated in the onset of PD which results in a loss of motor control (20). AD neurodegeneration begins in the entorhinal cortex (ETC) and then spreads to the hippocampus (19, 87), resulting in dementia. These two regions and the SN were selected to determine if mild CIH is capable of initiating inflammatory processes that may be responsible for neurodegeneration onset.

Oxidative stress (OS) and inflammation are hallmarks of neurodegeneration. An escalation in OS and inflammation has been documented in both clinical and animal models of severe OSA (79, 86). While many studies have looked at the inflammatory profile of the hippocampus after exposure to severe CIH (51, 72, 133, 147), the effect of mild CIH remains to be elucidated. In contrast to the volume of information regarding the hippocampus, this is the

first study to characterize the inflammatory profile of the SN and brainstem regions in response to CIH.

Oxidative stress is implicated in the progression of neurodegenerative diseases (9, 31), and is the result of the cell's inability to regulate reactive oxygen species (ROS). ROS, such as hydroxyl radicals, hydrogen peroxide, peroxynitrite, superoxide anion, play a critical role in signaling pathways under homeostatic conditions, but by their nature, are highly reactive and usually are short lived. OS accumulation can lead to protein dysfunction, creating a damaging environment within the cell. This environment, in turn, triggers apoptotic pathways and the release of the cytokine interleukin-1 β (IL-1 β) from the cell (93). We have previously published that OS increases with age in men (30). Interestingly, men have a significantly higher level of OS than women (48). This increase in OS in men may be related to the increased prevalence of some diseases, such as sleep apnea, in men (107, 112, 150). Furthermore, age-associated OS may be a major contributor to many age-related diseases.

Both OSA and CIH are associated with activation of angiotensin II (44) which is linked to OS through stimulation of NOX2 and other pathways (49, 155). Experiments which block the angiotensin II receptor (AT1aR) with losartan have demonstrated angiotensin II (ANG II) can interact with the CNS through ventricular organs such as the lamina terminalis (74, 75). Previous studies tracing the path of neuronal activation through FosB reactivity have implicated the median preoptic nucleus (MnPO) as a major contributor to the changes in mean arterial pressure (MAP) associated with CIH (28). Blocking AT1aR in the MnPO attenuates the sustained component of hypertension due to CIH (28). One mechanism contributing to elevated OS in CIH may be activation of the renin-angiotensin system through ANG II expression. Blocking the AT1aR in the MnPO may also reduce OS.

The increase in OS can increase the inflammatory response through IL-1 β , which is another hallmark of age-associated diseases. An increase in inflammation associated with OSA has been documented over the years (55, 86, 133). Chronic elicitation of inflammatory markers associated with OSA may be a contributing factor to the risk of neurodegeneration (16, 42, 56, 82, 94, 115, 129). In this study, we hypothesize that a 7 day CIH protocol is sufficient to increase OS, leading to a pro-inflammatory environment mediated through AT1a receptors and circulating blood-based biomarkers are predictive of the inflammatory state of the central nervous system.

Methods

Animals: All experiments were conducted according to National Institute of Health guidelines on laboratory animals. Experiments were approved by the Institutional Care and Use Committee at UNT Health Science Center. Adult Sprague-Dawley male rats (250-300g 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. All surgeries were conducted using aseptic techniques.

Chronic Intermittent Hypoxia (CIH): Male Sprague-Dawley rats (11-16) were housed in chambers that produce abrupt oxygen fluctuations over 6 minute cycles for 8 hours during the light phase to mimic the nocturnal hypoxic experience of patients with sleep apnea. Oxygen levels were reduced over 105 seconds to 10% and held for 75 seconds. Room air was then added to the chamber to increase oxygenation to 21% over 105 seconds and held another 75 seconds. This cycle continued for 7 days. For the remaining 16 hours, animals were exposed to room air. Control animals (7-11) resided in similar chambers using only room air (normoxic) for all cycles. Within 24 hours of the final CIH exposure, animals were deeply anesthetized and sacrificed as

previously described (28). Blood was collected in 7mL EDTA tubes and. 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 and flash frozen in PBS on dry ice then sliced into 1mm coronal sections. Brain nuclei from the rostral ventrolateral medulla (RVLM), solitary tract nucleus (NTS), substantia nigra (SN), entorhinal cortex (ETC), and hippocampus were isolated using a blunt 23 gauge needle with 1 mL syringes, immediately frozen on dry ice in 1.5mL polypropylene tubes, then stored at -80° C until homogenized for assays.

AT1a receptor knockdown: A subset of rats were administered a neurotropic adeno-associated virus (AAV) with shRNA for AT1a receptors through stereotaxic injection in the MnPO at 0.0-mm anterior, 0.9-mm lateral, and 6.7-mm ventral to bregma with the injector angled 8° from vertical prior to CIH exposure. Control animals were injected with scrambled shRNA. No difference in OS levels were found between untreated and scrambled, thus these groups were combined (figure 8).

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) and incubated on ice prior to sonication three times. Samples were then centrifuged 20 min at 12,000 x g at 4° C. The supernatant was extracted and transferred to a clean 1.5mL 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 and Western blotting protocols.

Advanced Oxidative Protein Products (AOPP) assay: Circulating oxidative stress (OS) was assayed using Cell Biolabs, Inc. OxiSelect Advanced Oxidative Protein Products assay kit, according to our previously published protocol (29).

Multiplexing: IL-10, IL-13, IL-4, IL-1 β , IL-6, TNF- α , KC/Gro, IFN- γ , IL-5, and IL-2 protein levels (table 1) in tissue homogenate samples were quantified using the Proinflammatory Panel 1 (rat) V-PLEX Kit from Meso Scale Diagnostics. This immunoassay allows for quantification 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 μ g of each tissue sample was quantified with the V-PLEX kit. Plasma cytokine levels were assessed using Bio-Rad's Bioplex Rat Th1/Th2 12-Plex kit and fluorescence measured on a Luminex platform. 35 μ L of each plasma sample was loaded into each well. Capture antibodies attached to magnetic beads react with each analyte of interest within each sample on a different region of the bead. Fluorescence in each bead region was detected and quantified following incubation with a streptavidin-phycoerythrin conjugate.

Western Blotting: Equal amounts (20 μ g) of protein were separated on a precast (any kD, 15 well comb, 15 μ g) polyacrylamide gel from Bio-Rad Laboratories, Inc. Proteins were transferred to polyvinylidene fluoride membrane overnight and incubated in primary antibodies for cd11b (Novus Bio, NB110-8947455, 1:500), cox-2 (Cayman Chemical, 160126, 1:250), nitrotyrosine (Cayman Chemical, 10189540, 1:250) overnight. Secondary antibodies (Santa Cruz, sc-2004, 1:2,000) were applied after washing for 1 hour. Chemiluminescent substrates from Thermo Scientific were used to induce luminescence. Images were captured with Alpha Innotech software (Alpha Innotech v. 1.2.0.1 Alpha Innotech Corporation, 2008) on an Alpha Innotech imager (Cell Biosciences). Protein was normalized to GAPDH protein expression and

densitometry quantified with Image J (v. 1.48, Wayne Rasband, National Institutes of Health, USA)

Statistical analysis: Assay results are reported as percent of control (individual value/(average of control values) x 100). IBM SPSS (SPSS v. 21 IBM, 2012) was used for statistical analysis. Independent T-tests assessed the difference between the means of AOPP and cytokines by treatment and Pearson's correlations were applied to correlate associations between oxidative stress and inflammatory markers. Results are shown as mean \pm SEM, and were considered statistically significant at $p < 0.05$.

Results

Plasma AOPP levels, a measure of OS, were normalized to controls and expressed as a percentage of control. CIH AOPP levels (n=11) were compared to the control AOPP levels (n=8). Figure 9 shows OS in plasma is significantly elevated in animals exposed to 7 days of CIH compared to controls. ($t=3.863 \pm 0.072$, $p=0.001$)

Cytokine levels in plasma were assessed to determine the inflammatory response to CIH (table 1). Following CIH exposure (n=11), all circulating cytokines of interest were increased significantly, except TNF- α and KC/Gro (table 2). IL-4, IL-1 β , IL-6, and IFN- γ levels in CIH animals were all observed to be more than 2 times the level of control animals. Particularly, IL-1 β levels in the CIH condition ranged from 2.7% - 44.0% higher than all other cytokines.

A significant association between OS (as indicated by AOPP) and inflammation was observed. This indicates an increase in oxidative stress is associated with an increase in inflammation (table 3). All cytokines of interest were significantly positively associated with AOPP levels ($p < 0.05$)

It is possible that other factors may contribute to the observed inflammatory increase, so associations with IL-1 β were also assessed. IL-1 β was selected due to the extremely high signal observed and its known function as a primary inflammatory signal. As seen on table 4, all correlations to IL-1 β were extremely strong and highly significant ($p < 0.0005$).

The hypoxemia experienced by OSA patients leads to an increase in circulating ANG II (1). An increase in neural activity in the median pre-optic nucleus has been observed in association with ANG II (28), so AT1a receptors were knocked down through the injection of a shRNA expressing adenovirus into the MnPO to ascertain what effects this may have on OS. Interestingly, AOPP levels in the plasma of the subset receiving the AT1a receptor virus were not significantly elevated in the CIH group (Figure 10). Similarly, table 5 illustrates that the inflammatory cytokine levels were not different from controls and all correlations observed in the untreated groups were lost. ANG II may contribute to circulating OS and inflammation through neuronal processes.

Within brain nuclei, a significant decrease in IL-10 ($t = 2.167 \pm 21.986$, $p = 0.048$), IL-4 ($t = 2.877 \pm 19.818$, $p = 0.012$), IL-6 ($t = 2.212 \pm 14.107$, $p = 0.044$), and TNF- α ($t = 2.335 \pm 18.959$, $p = 0.035$) in the RVLM, a brainstem nucleus, was observed in animals exposed to CIH (table 6). A decreasing trend of nitrosylated proteins in the RVLM ($t = 2.238 \pm 16.738$, $p = 0.060$) was also observed (figure 11A & B). There was not a significant difference in microglial activation or COX2 expression within this nucleus. No significant differences in cytokines (table 6) or protein expression (data not shown) was observed in the NTS.

CIH significantly increased CD11b ($t = -3.567 \pm 54.519$, $p = 0.023$), an integrin found in activated microglia, in the SN of animals. (figure 11C & D). No differences were observed in

Cox2 expression, a protein involved in inflammatory apoptotic pathways, or NT in the SN after 7 days of CIH. Although no significant changes in cytokine levels were observed in the SN, a significant association between OS with KC/Gro ($r^2 = 0.323$, $p = 0.027$) and TNF- α levels ($r^2 = 0.383$, $p = 0.014$) was noted (figure 12A & B).

In the ETC, a region with projections to the hippocampus that is implicated in early cognitive impairment (19), there was a trend of elevated KC/Gro ($t = -2.144 \pm 21.668$, $p = 0.074$; table 6). Circulating OS was significantly associated with KC/Gro levels within the ETC ($r^2 = 0.547$, $p = 0.006$; figure 12A). No significant differences in protein expression within the ETC were observed (data not shown).

A significant decrease in NT, an indicator of OS, was observed in the hippocampus ($t = 4.134 \pm 18.179$, $p = 0.014$; figure 11E & F). There were no significant differences in the inflammatory response to CIH observed in hippocampal tissue by either immunoassay or western blot analysis (table 6, figure 11).

Discussion

As observed in this present study, mild to moderate CIH is capable of producing systemic/peripheral inflammation and OS, similar to effects found in severe CIH models (55, 79, 86). Oxidative stress levels were significantly elevated following 7 days of CIH (figure 9). This was accompanied by an elevated peripheral inflammatory response to CIH (table 2). This suggests, left untreated, a lifetime of chronic intermittent hypoxia, as experienced in OSA, may contribute to an accumulation of OS within a patient. Unchecked, this can initiate or exacerbate metabolic conditions leading to disease. Treatments, such as CPAP, which alleviate the

hypoxemia experienced in OSA could be beneficial to prevent the accumulation of OS as a patient ages as well as promote longevity and a higher quality of life.

Increased OS was accompanied by a distinct increase in inflammation (table 3). IL-1 β levels were observed to be more than two times basal levels when exposed to CIH. IL-1 β is stored as a pro-form within cells and is released upon cellular insult to initiate signaling cascades in many inflammatory responses. Its primary purpose serves to recruit initial responses to injury or infection, activating lymphocytes and making vascular walls more permeable. We were interested to see if any of the cytokines were correlated to the elevated IL-1 β levels. Linear regression models show a highly significant positive relationship with IL-1 β for all the cytokines measured (table 4). It is possible elevated IL-1 β is a major contributor to the significant increase in inflammation observed in CIH models.

Of particular interest is the significant correlation between OS and TNF- α . TNF- α was not significantly elevated in response to the CIH treatment, but is significantly positively associated with an increase in OS. TNF- α is known to trigger classical symptoms of inflammation and can lead to systemic shock in large amounts (93). Consequently, there are many mechanisms in place to regulate TNF- α within a healthy body. It is conceivable 7 days of CIH is not long enough to overcome these checkpoints, but a chronic accumulation of OS may eventually increase TNF- α levels to the degree tissue damage is sustained. This is one more incentive to reduce OS levels in patients prone to age-related diseases.

Interestingly, knocking down neuronal AT1a receptors in the MnPO attenuated the observed increase in OS (figure 10) and inflammation (table 5). This corroborates prior studies which point to a crucial role for ANG II in the production and accumulation of ROS that can

contribute to disease (49, 155) (figure 13). It would be valuable to further study the role ANG II plays in metabolic diseases associated with increased circulating OS and aging. The involvement of a neural component involved in ANG II signaling and the established association with cognitive deficits experienced in conjunction with CIH (97) suggests further study is needed investigate the role ANG II may play in initiating neuronal disorders.

Based on our results, the initial impact of mild CIH on brain nuclei appears to vary depending on the function of those nuclei. This is in agreement with other studies which show differential responses in mRNA expression and inflammatory markers by region and length of exposure to CIH (53, 121, 133).

Because projections directly to the brainstem are accessible to systemic inflammatory and OS signals, which are elevated by CIH, a prevailing opinion is the RVLM and NTS would most likely exhibit an increase in cytokines in response to systemic stimulation (32, 37). Indeed, systemic application of LPS has been demonstrated to have a direct impact on the RVLM (146) and physiological effects of CIH have been linked to altered sympathetic control through brainstem structures (1, 28, 89). However, other studies have found that intrinsic electrochemical properties of neurons within the RVLM are not altered by CIH exposure (2), demonstrating the dichotomous roles these nuclei can play in maintaining physiological homeostasis (66, 69).

According to our results, the 7 day response to CIH appears to be protective within the RVLM and CIH had no effects on the NTS. Although Smith, et al (133) observed a significant increase in mRNA expression for IL-1 β , IL-6, TNF- α , COX2, and iNOS within brainstem homogenates of rats exposed to CIH (AHI=15), we observed a significant suppression of inflammatory cytokine proteins (table 6), no significant difference in COX2 protein expression, and a trend of decreasing NT protein in the RVLM (figure 11A & D). This reduction in

inflammation may be symptomatic of non-damaging effects by 7 days of mild CIH exposure to maintain homeostatic physiological functions within the RVLM, while more severe exposures are detrimental. Additionally, mRNA expression may not always be indicative of actual protein levels if post-transcriptional modifications prohibit the translation of mRNA into protein. Future studies may include both protein and mRNA analysis to address this discrepancy.

Although sleep apnea is often a comorbidity associated with PD (4, 24, 125), and an elevation of both OS and inflammation are hallmarks of PD (9, 23, 67, 116), we were unable to locate research showing causality between these conditions. Our results show a significant increase in activated microglia within the SN, the nuclei associated with PD (20) (figure 11B). This is accompanied by a positive correlation of KC/Gro and TNF- α in the SN with plasma OS levels (figure 12A &B), wherein as OS increased, KC/Gro and TNF- α increased. KC/Gro is associated with recruitment of granular cells and increased membrane permeability (90). Therefore, in the SN, circulating OS is associated with increased inflammatory markers.

Similar to the SN, KC/Gro in the ETC is also positively associated with plasma OS levels (figure 12C). Because the ETC is one of the first areas impacted in the development of AD (19), this suggests that systemic OS may be a contributor towards and be indicative of the inflammation associated with initial cognitive dysfunction.

Because OSA has been linked to cognitive impairments in patients (45, 105), severe CIH models have explored and demonstrated altered hippocampal structure and learning deficits (51), and treatment of OSA leads to improved cognitive function (139), OSA may be a contributing factor to the development of AD. Although we did not see significant alteration in the inflammatory profile of hippocampal homogenate of animals exposed to 7 days of mild CIH, it is

possible the onset of neurodegeneration in this area due to OSA could happen at a later time point. This is in agreement with the pathophysiology of AD (19) in which marked degradation occurs first in the ETC, and then progresses into the hippocampus. Our data supports this progression, wherein we observed a KC/Gro and OS association in the ETC. Additionally, Gozal, et al, 2003 (53) observed an initial elevation in apoptotic cells within the hippocampus after 1 day of severe CIH exposure that had returned to normal levels by days 7 and 14. Our results observed in the hippocampal tissue at 7 days would be consistent with their more severe exposure.

Nitrosylation of proteins has been shown to be an indicator of OS. The reaction between superoxide and nitric oxide (NO) forms peroxynitrite that can cause nitrosylation of tyrosine residues of proteins in the cytosol. While nitrotyrosine (NT) is normally present within controls, a higher NT signal can be observed within tissue under OS (79). CIH has been associated with an initial decrease in the availability of NO followed by an over production of NO at later time points as inducible nitric oxide synthase becomes activated (155). The significant reduction of NT observed in the hippocampus (figure 11) may be reflective of the reduced bioavailability of NO necessary to form peroxynitrite after 7 days CIH, similar to what is observed in endothelial cells (85). It remains to be seen if a longer exposure would trigger an elevation in NT through inducible nitric oxide synthase, as is typical in more severe hypoxic exposures (79).

It appears that while systemic inflammation associated with mild CIH can be protective in nuclei associated with homeostatic functions, such as the RVLM, it initiates inflammatory pathways in regions associated with neurodegenerative diseases. This study's results indicate that mild to moderate levels of hypoxia can impact physiology. While it may be tempting from these results to consider administration of pharmaceutical treatments which block the effects of ANG

II in patients with OSA, it is important to remember ANG II plays a vital role in other homeostatic functions, such as regulation of blood pressure (1, 31), maintenance of bone density (111, 130), and production of reproductive hormones (36, 60). Additionally, selective administration of a shAT1a AAV into the MNPO of a patient would be highly invasive and long term effects of these injections have not been studied. These results should serve more to suggest taking preventative steps to reduce and prevent accumulation of OS by CIH as early as possible may be wise. Furthermore, It may be possible to use circulating OS levels and inflammation as early-stage biomarkers for determining populations at risk for neurodegeneration.

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FIGURE 8

AOPP LEVELS OF UNTREATED AND SCRAMBLE CONTROL ANIMALS

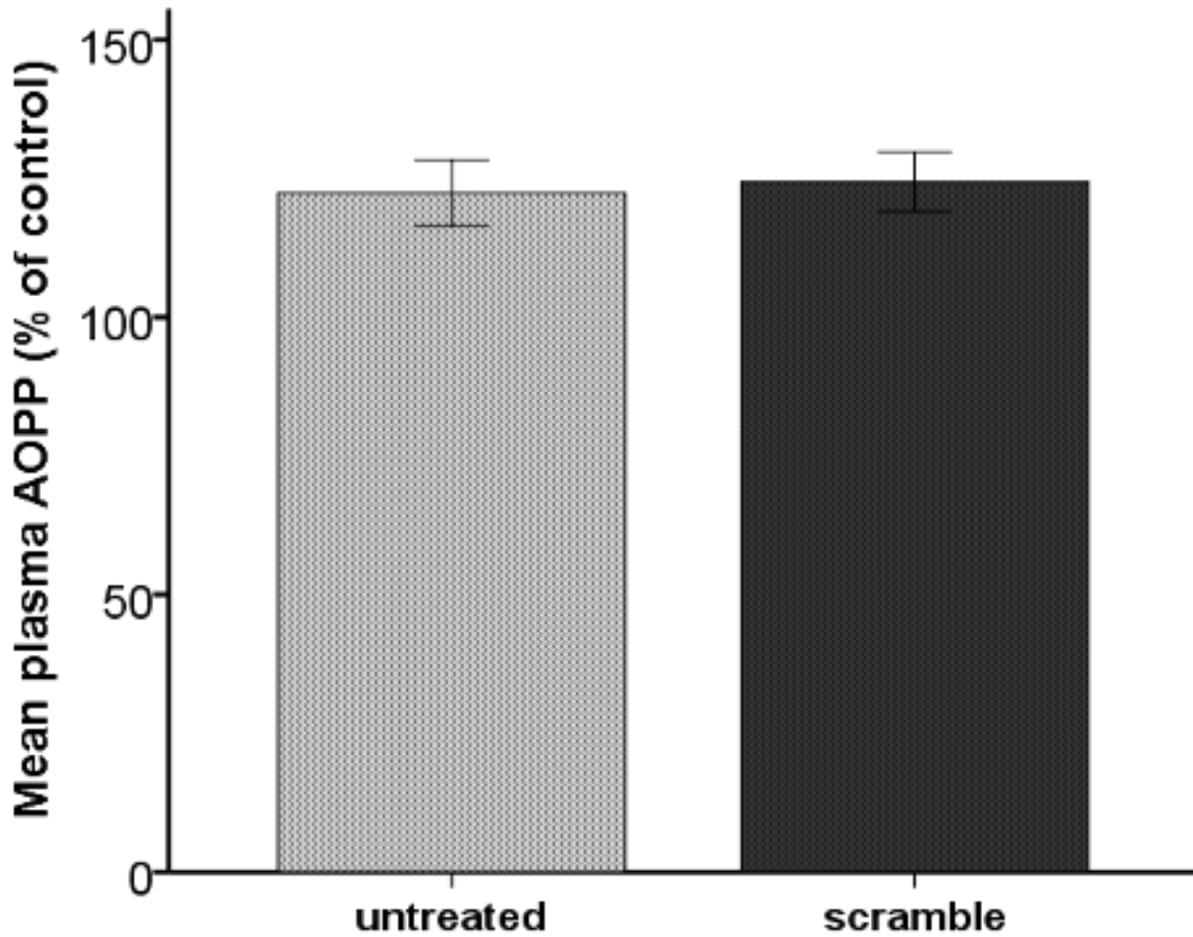


Figure 8 Administration of the scramble virus has no effect on circulating AOPP levels (p=0.826)

FIGURE 9

PLASMA AOPP LEVELS OF ANIMALS EXPOSED TO CIH

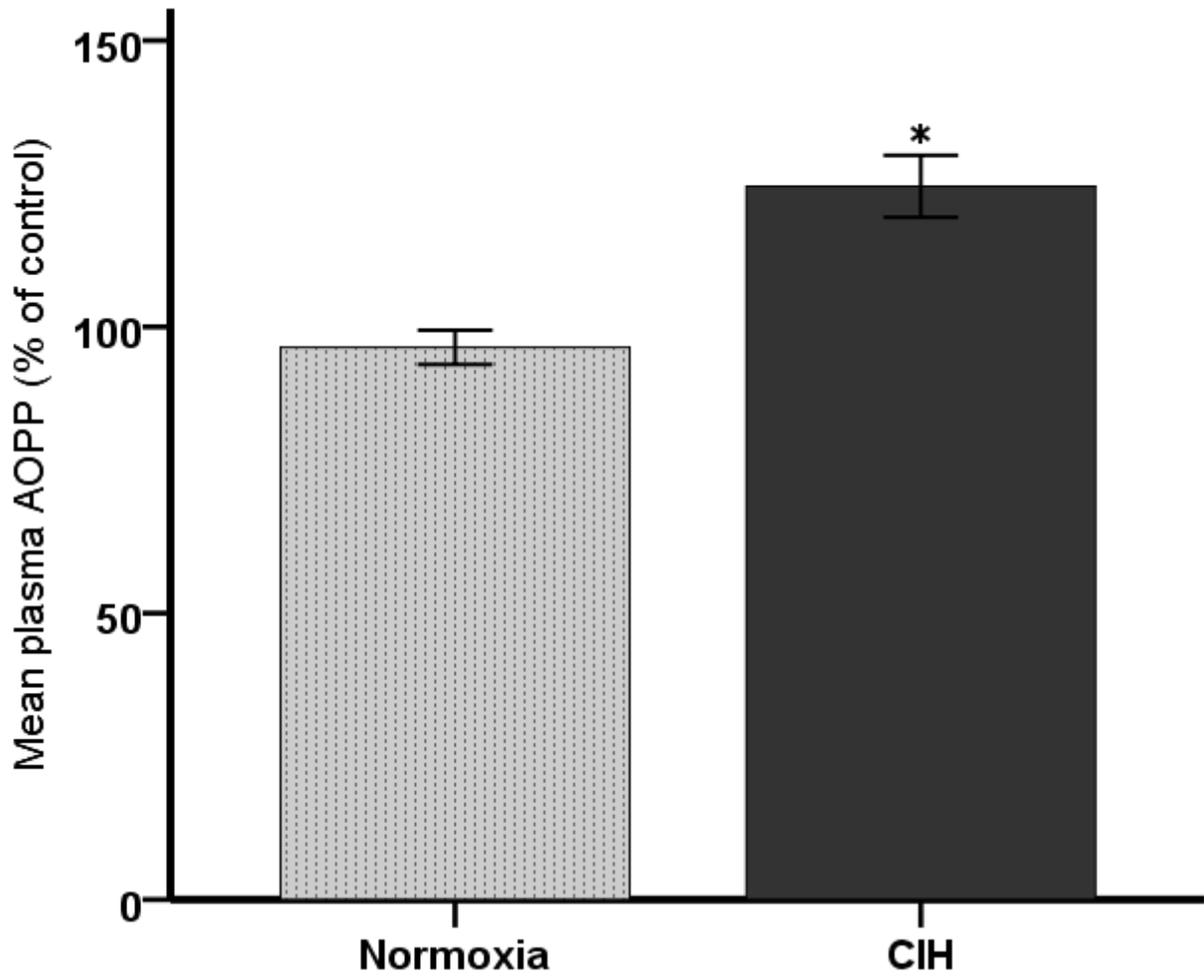


Figure 9 7 day exposure to CIH significantly increases circulating OS in male rats. (p=0.001)

FIGURE 10

AOPP LEVELS OF ANIMALS RECEIVING THE shAT1a ADENOVIRUS IN THE MnPO

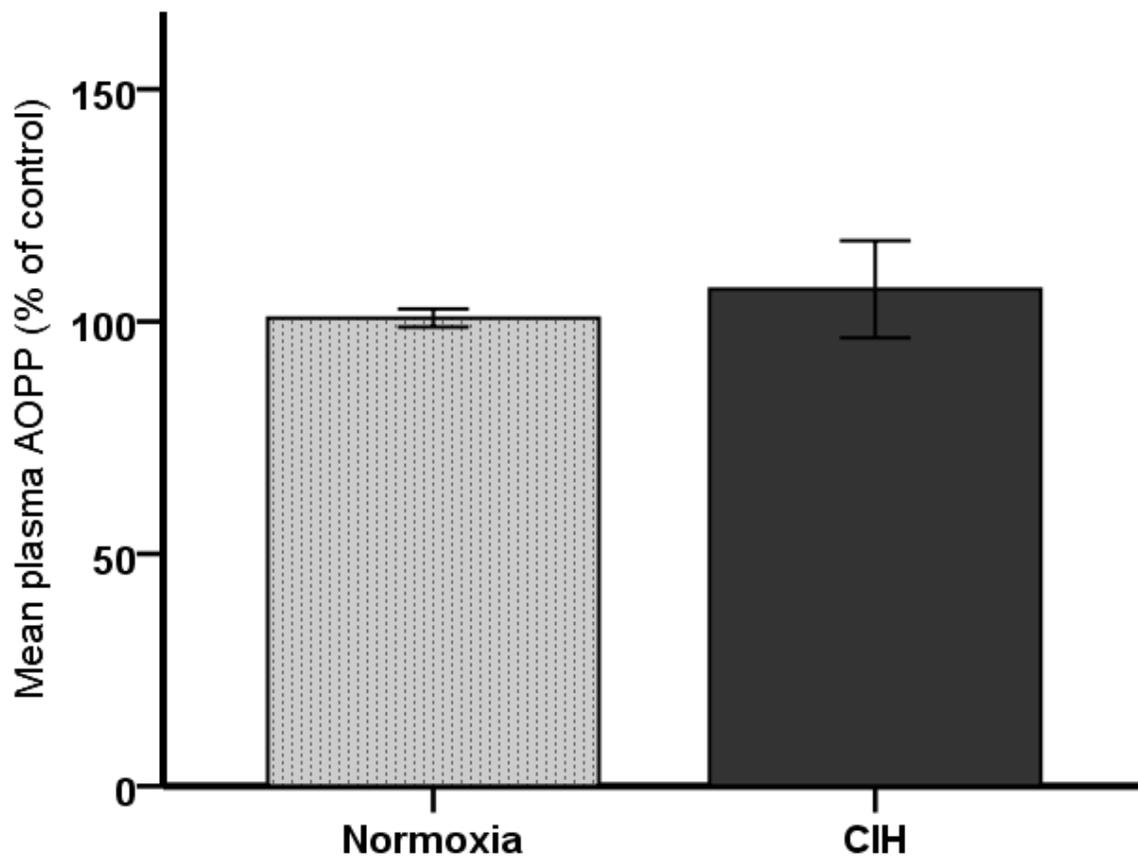


Figure 10 Administration of the sfAT1a receptor virus prevents the previously observed increase in AOPP levels during 7 days of CIH. ($p = 0.573$)

FIGURE 11

PROTEIN EXPRESSION IN BRAIN REGIONS ASSOCIATED WITH NEURODEGENERATIVE DISEASES

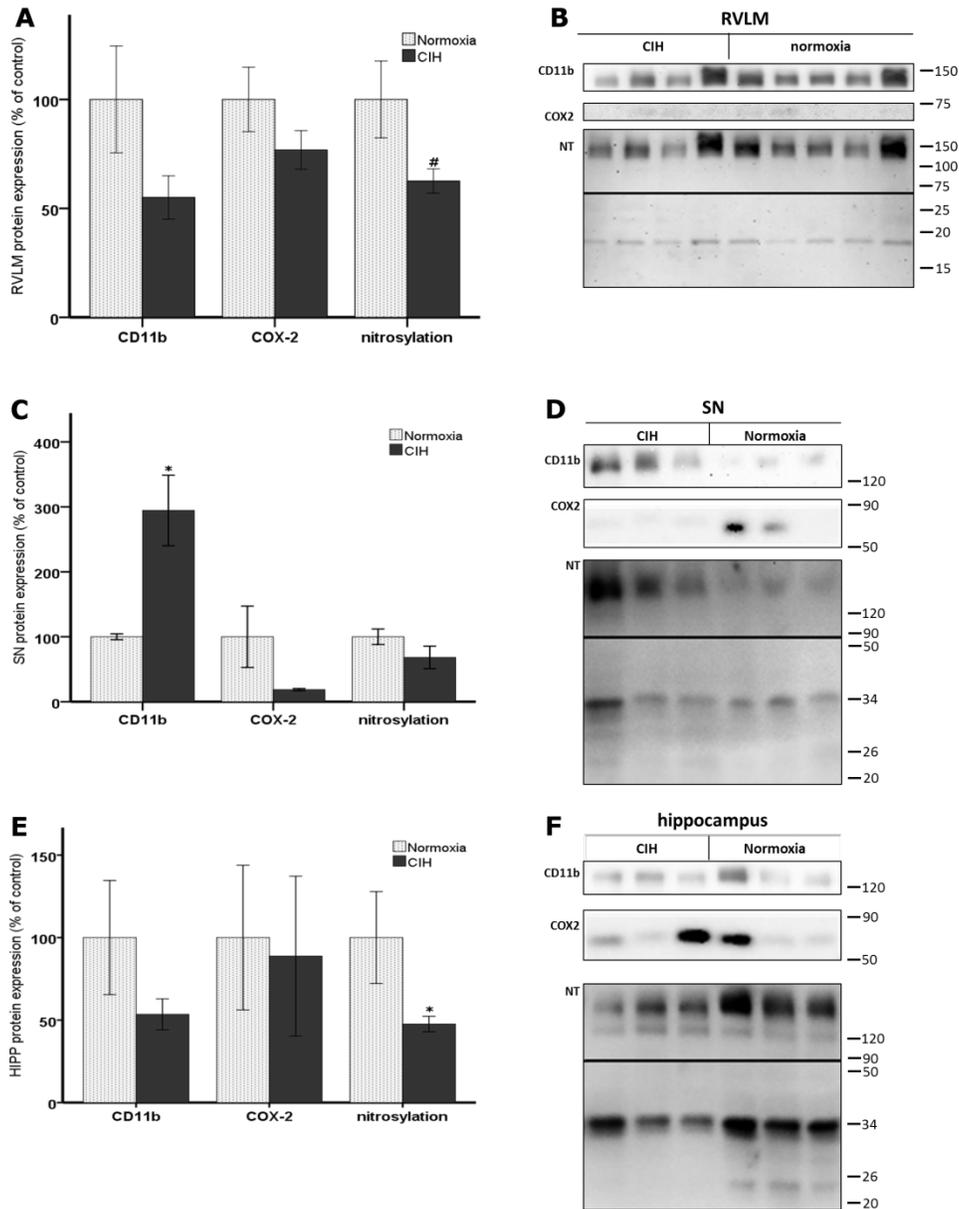


Figure 11 Protein analysis by Western blotting within different brain regions shows variability in protein expression by nuclei. Within the RVLM, a trend of decreasing NT ($p = 0.060$) is also observed (A, B). A significant increase in CD11b ($p = 0.023$) in the SN is observed in animals subjected to 7 days mild CIH (C, D). Simultaneously, there is a significant decline in NT ($p = 0.014$) in the hippocampus of male rats undergoing CIH (E, F)

FIGURE 12

REGIONAL CYTOKINE EXPRESSION CORRELATED TO AOPP

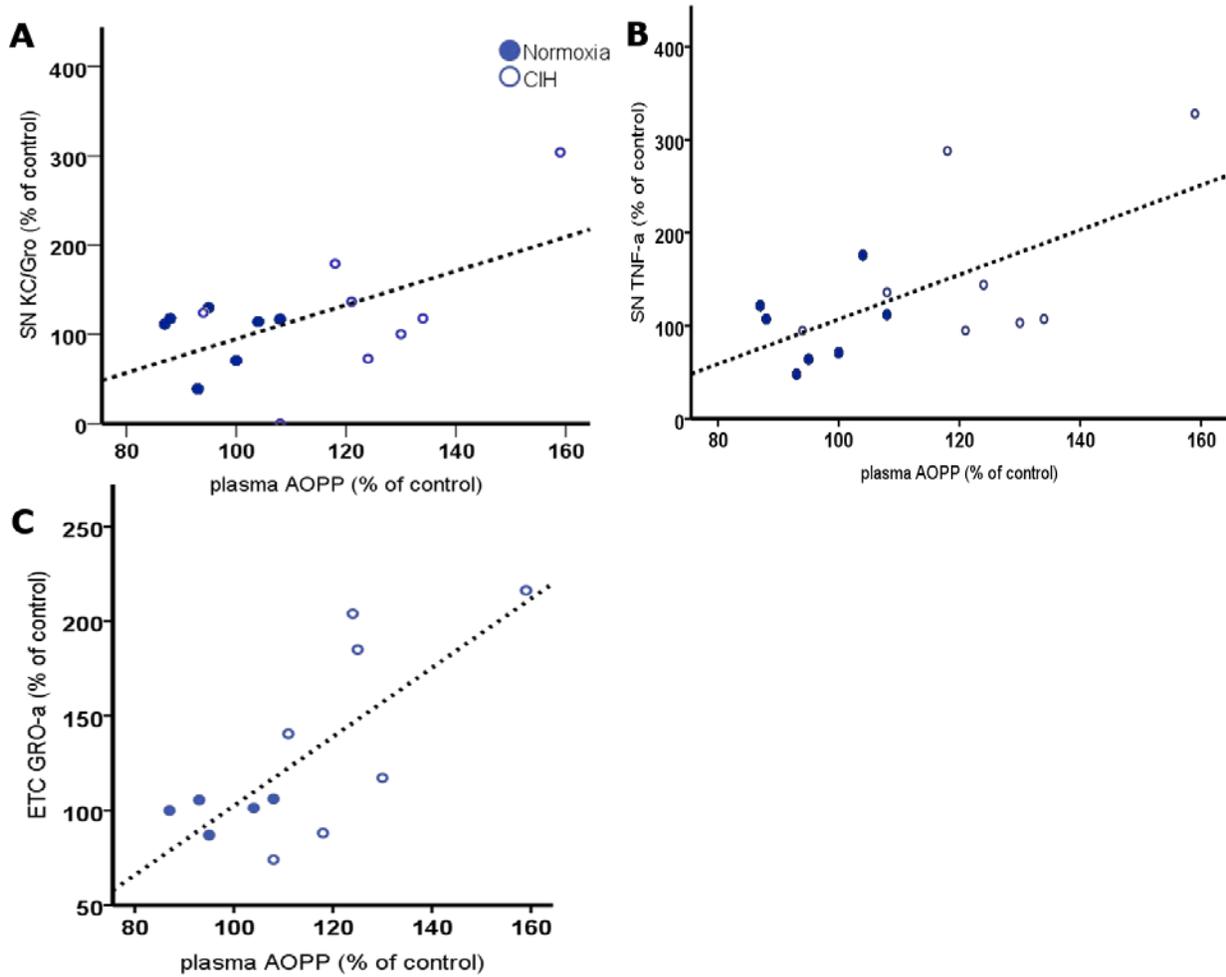


Figure 12 Circulating OS, as measured by AOPP, is associated with inflammatory cytokines in areas associated with neurodegeneration. OS is positively associated with KC/Gro ($r^2 = 0.323$, $p = 0.027$) (A) and TNF-a ($r^2 = 0.383$, $p = 0.014$) (B) in the SN. In the ETC, KC/Gro is also positively associated with OS ($r^2 = 0.547$, $p = 0.006$) (C).

FIGURE 13

CIH INDUCTION OF OS & INFLAMMATION MODEL

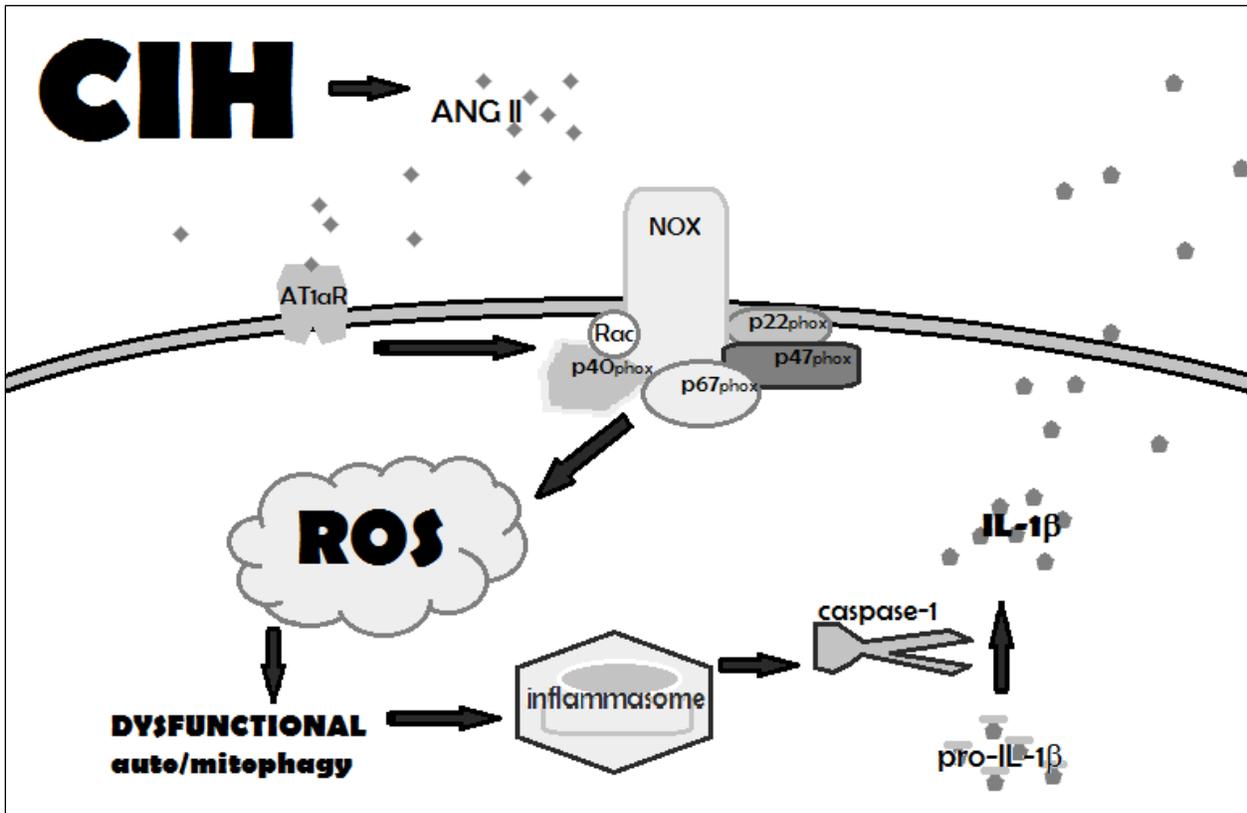


Figure 13 Proposed model illustrating how CIH experienced by patients with sleep apnea may lead to an inflammatory environment that lends itself to the development of age-related diseases.

TABLE 1

CYTOKINES OF INTEREST

cytokine	microglia	neuron	pro-	anti-
<i>IL-10</i>	+	-	+	+
<i>IL-13</i>	+	-	-	+
<i>IL-4</i>	+	-	-	+
<i>IL-1β</i>	+	+	+	-
<i>IL-6</i>	+	-	+	-
<i>TNF-α</i>	+	+	+	-
<i>KC-Gro</i>	+	-	+	-
<i>IFN-γ</i>	+	-	+	+
<i>IL-5</i>	+	-	+	+
<i>IL-2</i>	+	-	+	+

Table 1 Cytokines of interest for this study and known role in neuroinflammation (82, 90, 99, 114, 118, 131, 154)

TABLE 2

PLASMA CYTOKINE EXPRESSION DUE TO CIH EXPOSURE

<i>cytokine</i>	Normoxia (n=7)	CIH (n=11)	p-value
<i>IL-10</i>	99.71 ± 7.18 (473.30 ± 35.08)	147.24 ± 12.74 (690.57 ± 186.74)	0.013*
<i>IL-13</i>	96.23 ± 8.78 (119.03 ± 10.23)	181.74 ± 17.68 (230.31 ± 56.30)	0.002**
<i>IL-4</i>	98.17 ± 10.42 (51.83 ± 5.73)	203.62 ± 27.11 (105.32 ± 23.92)	0.003**
<i>IL-1β</i>	95.10 ± 12.74 (883.97 ± 116.90)	200.25 ± 24.29 (1870.30 ± 13.34)	0.002**
<i>IL-6</i>	98.03 ± 16.82 (67.32 ± 11.54)	221.14 ± 32.49 (152.68 ± 228.67)	0.012*
<i>TNF-α</i>	105.61 ± 15.12 (59.86 ± 10.99)	167.92 ± 25.22 (96.44 ± 22.65)	0.086
<i>KC/Gro</i>	100.76 ± 9.61 (34.76 ± 4.15)	143.78 ± 20.70 (47.37 ± 15.14)	0.081
<i>IFN-γ</i>	97.37 ± 21.91 (76.54 ± 17.04)	222.62 ± 34.27 (172.43 ± 25.87)	0.016*
<i>IL-5</i>	97.33 ± 6.40 (278.58 ± 19.67)	143.77 ± 7.28 (65.25 ± 19.67)	0.000**
<i>IL-2</i>	97.25 ± 10.64 (304.32 ± 33.13)	165.58 ± 12.51 (133.57 ± 40.27)	0.002**

Table 2 Circulating cytokines increase significantly during 7 days of CIH. Bold values are presented as mean fluorescence ± SEM normalized to controls. Values in italics are actual mean fluorescence per group.

*Results are considered significant when $p < 0.05$. ** $p < 0.01$

TABLE 3

CYTOKINE vs. AOPP CORRELATION

<i>Cytokine v. AOPP</i>	Pearson Correlation (r) (n=18)	p-value
<i>IL-10</i>	0.426	0.039*
<i>IL-13</i>	0.528	0.012*
<i>IL-4</i>	0.521	0.013*
<i>IL-1β</i>	0.538	0.011*
<i>IL-6</i>	0.481	0.022*
<i>TNF-α</i>	0.55	0.009**
<i>KC/Gro</i>	0.084	0.369
<i>IFN-γ</i>	0.501	0.017*
<i>IL-5</i>	0.554	0.008**
<i>IL-2</i>	0.612	0.003**

Table 3 Circulating inflammatory markers show a significant positive association with AOPP levels.

*Results are considered significant when $p < 0.05$. ** $p < 0.01$

TABLE 4

CYTOKINE vs. IL-1 β CORRELATION

<i>Cytokine v. IL-1β</i>	Pearson Correlation (r) (n=18)	p-value
<i>IL-10</i>	0.94	0.000***
<i>IL-13</i>	0.982	0.000***
<i>IL-4</i>	0.945	0.000***
<i>IL-6</i>	0.93	0.000***
<i>TNF-a</i>	0.756	0.000***
<i>KC/Gro</i>	0.026	0.519
<i>IFN-g</i>	0.839	0.000***
<i>IL-5</i>	0.952	0.000***
<i>IL-2</i>	0.949	0.000***

Table 4 Circulating inflammatory markers show a highly significant positive association with IL-1 β levels.

*Results are considered significant when $p < 0.05$. ** $p < 0.01$ *** $p < 0.005$

CYTOKINE EXPRESSION OF ANIMALS DUE TO CIH EXPOSURE FOLLOWING
shAT1a INJECTION

TABLE 5

<i>cytokine</i>	Normoxia (n=7)	CIH (n=11)	p-value
<i>IL-10</i>	98.52 ± 16.15	100.25 ± 15.03	0.939
<i>IL-13</i>	96.33 ± 26.26	90.43 ± 4.09	0.812
<i>IL-4</i>	98.05 ± 12.73	85.36 ± 10.15	0.456
<i>IL-1β</i>	97.52 ± 25.24	91.47 ± 9.32	0.814
<i>IL-6</i>	95.65 ± 12.71	100.70 ± 17.95	0.842
<i>TNF-α</i>	87.99 ± 22.27	99.50 ± 12.00	0.632
<i>IFN-γ</i>	97.41 ± 26.36	89.59 ± 16.31	0.742
<i>IL-5</i>	92.40 ± 18.98	97.31 ± 7.01	0.800
<i>IL-2</i>	97.95 ± 19.70	108.88 ± 38.05	0.669

Table 5 Administration of the shAT1a virus in the MNPO attenuates the inflammatory response to CIH. No cytokine was found to significantly alter from controls during CIH treatment. Values are presented as mean fluorescence ± SEM normalized to controls.

*Results are considered significant when $p < 0.05$.

CYTOKINE FLUORESCENCE IN TISSUE SAMPLES BY BRAIN REGION

cytokine	RVLM		SN		ETC		Hippo		NTS	
	CIH (n=9)	p-value	CIH (n=8)	p-value	CIH (n=7)	p-value	CIH (n=11)	p-value	CIH (n=11)	p-value
IL-10	52.36 ± 12.92	0.048*	78.31 ± 21.27	0.447	65.34 ± 32.29	0.168	102.88 ± 25.94	0.939	58.60 ± 14.97	0.171
IL-13	68.64 ± 12.98	0.104	64.31 ± 20.37	0.255	97.56 ± 30.59	0.927	81.06 ± 18.70	0.648	51.63 ± 13.96	0.404
IL-4	42.98 ± 11.84	0.012*	192.51 ± 104.16	0.445	71.52 ± 22.77	0.261	105.31 ± 31.95	0.917	63.13 ± 15.20	0.292
IL-1β	79.42 ± 13.66	0.215	81.74 ± 15.17	0.391	100.94 ± 24.90	0.945	77.14 ± 9.39	0.134	79.14 ± 17.01	0.406
IL-6	68.79 ± 10.77	0.044*	126.19 ± 17.01	0.339	116.81 ± 41.87	0.453	80.81 ± 15.79	0.368	70.29 ± 16.54	0.411
TNF-α	55.73 ± 12.02	0.035*	162.02 ± 32.71	0.129	78.64 ± 37.89	0.337	92.07 ± 22.00	0.783	65.09 ± 17.17	0.226
KC/GRO	101.98 ± 11.38	0.910	129.33 ± 31.04	0.421	146.45 ± 56.59	0.074	72.61 ± 13.28	0.184	125.39 ± 20.26	0.427
IFN-γ	77.94 ± 9.44	0.121	99.22 ± 11.91	0.960	109.66 ± 21.09	0.481	91.04 ± 15.58	0.662	74.67 ± 9.72	0.106
IL-5	94.82 ± 22.59	0.846	90.46 ± 22.32	0.751	98.08 ± 34.44	0.946	81.95 ± 19.10	0.572	77.04 ± 16.47	0.464

Table 6 Mean fluorescence of cytokines within each nucleus. Values are expressed as percent of control ((individual fluorescence/mean of control fluorescence) x 100) *Results are considered significant when p<0.05.

TABLE 6

CHAPTER III

DISCUSSION

Neurodegenerative diseases (ND) such as Alzheimer's disease (AD) and Parkinson's disease (PD) are a major health concern. Currently, there are not effective pharmaceutical treatments to prevent progression of either of these diseases (11, 77). Current treatments temporarily alleviate some symptoms but do not cure or halt ND (11). Major clinical trials of proposed treatments which appeared promising in animal models have failed to produce satisfactory results. The current cost of treatment for a patient with AD is \$46,669/year (68) and \$22,800/year for a patient with PD (68, 77). Both the number of cases and the financial burden of these diseases are expected to rise dramatically by 2050 (68, 77).

Neurodegeneration progresses in each type of disease in a predictable pattern. Pathological progression of neurodegeneration in AD has been documented to initiate in the entorhinal cortex (ETC) in a manner consistent with clinical features of mild cognitive impairment (19, 70). Neuronal apoptosis and formation of plaques advance to the hippocampus and finally impact the frontal cortex as well in later stages. Neurodegeneration in PD, on the other hand, appears to initially impact the dopaminergic neurons of the substantia nigra pars compacta (SN), leading to a loss of inhibition of motor neurons in the striatum. Associated with this loss is an accumulation of α -synuclein deposits known as Lewy bodies (20).

Two hallmarks characteristic of ND are oxidative stress (OS) and inflammation. The mechanisms which elicit these signals have been a focus of research endeavors and reviews in

recent years (7, 23, 42, 82, 90, 94, 108, 153). Identifying the cause of elevated inflammation in brain tissue and the mechanisms that predispose one region to be more profoundly impacted than another between individuals would provide potential therapeutic pathways to prevent cognitive decline in AD or further loss of motor control in PD. Because of the similarity of characteristics, it is possible one type of stimulus provides the OS and inflammatory environment and genetic and environmental factors contribute to the tissue of pathological impact. OS contributes to inflammation through activation of the nuclear factor (NF-kB) pathway (86). Both circulating OS and inflammatory markers have been documented to impact gene expression in neurons.

There are sex differences within ND. Men are twice as likely to develop PD as women are (25, 46). Prior to menopause, men are also more likely to be diagnosed with AD (91). After this point, diagnosis of AD is equivalent between the sexes until 78-80 years old (91). Measurements of cortical activity and hormone levels in the brain are very different between men and women with AD, suggesting the paths which lead to neurodegeneration are varied and progress in a sex-specific manner (91, 119). For this reason, research investigating factors that have a sex-bias may be necessary to elucidate appropriate interventions. It is possible personalized prevention and treatment may require a multi-faceted approach involving the identification and treatment of early risk factors (11).

The occurrence of sleep disturbances and comorbidity of obstructive sleep apnea (OSA) within patients with ND has been well documented (4, 24, 50, 73, 102, 125, 139, 142). Because OSA and ND share many characteristic symptoms, there is speculation as to the role OSA contributes to the initiation of ND. Further investigation into mechanisms by which OSA may contribute to neurodegeneration is necessary to establish a causal relationship. This thesis

presents results suggesting the chronic intermittent hypoxia (CIH) experienced by patients with OSA may be one mechanism impacting ND in men.

OSA is on the rise in western society (41, 88, 107, 150), possibly due to its association with age and BMI. As the BMI of our population increases as well as the average age, the prevalence of OSA may rise in the coming years. Increasing popular awareness of the impact OSA has on health may also contribute to a higher number of patients.

OSA has been linked to cognitive and motor dysfunction in prior studies (12, 34, 45, 50, 73, 78, 79, 105, 148). Treatment of OSA by continuous positive airway pressure (CPAP) in patients co-diagnosed with AD has demonstrated the ability to prevent further cognitive decline over a period of years and improves functional memory assessment results to some degree (45, 102, 139). Recovery of motor skills following OSA has shown equivocal results (45, 78). There may be some neuronal regions which are impacted irreversibly by CIH, which makes early diagnosis and retention of these neurons all the more urgent. In fact, there is evidence of neurogenesis following CIH in hippocampal structures that has not been observed to date in the substantia nigra (53). In severe models of OSA, there is evidence of recovery of cognitive functions once pathways induced by the CIH stimulus are suppressed (35, 52, 64, 96). The ability to have differential effects and replenish neurons in a region specific manner following CIH insult is an area that deserves further research. To date, studies have mainly explored the effects of severe CIH exposure, with only cursory data about its impact in differing central nervous system (CNS) regions.

Because many more patients have mild – moderate OSA, this study focused on the impact early exposure to mild CIH might have on brain regions associated with various functions. Mild CIH has been documented to generate systemic effects in male rats similar to

what is observed in more severe models (28, 44, 74). It is conceivable mild CIH may have similar central effects as well.

OS is elevated in both clinical and animal models of ND and severe OSA (9, 30, 62, 67, 79, 103, 147). Reactive oxygen species (ROS) function as signaling molecules and maintain health at normal homeostatic levels (22, 66, 128, 140, 141, 149). Toxicity occurs when ROS accumulate beyond the redox capabilities of the cell, reaching levels referred to as oxidative stress (OS) (14, 31, 93, 128). OS is capable of elevating inflammatory and apoptotic pathways (93). It has been suggested that consumption of known antioxidants may be able to restore homeostatic status, but trials utilizing antioxidants have been ineffective in modulating OS in ND to date (109). Therefore, it may be more worthwhile to identify pre-clinical conditions which contribute to OS accumulation and treat those in an effort to slow the natural accumulation of ROS over the aging process (31, 79, 109, 140). In this study, circulating OS was observed to be significantly elevated in male rats subjected to 7 days mild CIH (AHI = 10) when compared to levels of OS in male rats exposed to normal room air oxygen (21%) (figure 8). This concurs with prior studies which have documented an increase in OS in CIH models.

Prior research into the mechanisms of neural contributions to the systemic effects of CIH has identified a role of the circumventricular organs and the brainstem in the regulation of autonomic functions during CIH (74, 100). Increased neural activity, as indicated by Δ FosB expression, was identified in the median preoptic nucleus (MnPO) of the hypothalamus as well as in the RVLM, and NTS (74). Blocking this expression prevented occurrence of systemic effects. Angiotensin II (ANG II) is elevated in CIH and is documented to contribute to the systemic effects (44). It is well known for acting upon angiotensin II receptors (AT1aR) to

modulate hypertension. ANG II elevates OS through these same receptors by activating NOX2 transcription (49, 155).

A subset of the rats in this experiment were injected with an AT1aR adenovirus into the MnPO to selectively inhibit the receptors in that region and characterize the role it plays in central nervous system control during CIH. The rats receiving the injection prior to CIH exposure did not experience a systemic elevation of OS as observed in the rats without the injection (figure 10). The complete abrogation of OS elevation suggests a major role for the neural components activated by CIH exposure.

Two mechanisms have been suggested as a means for elevated systemic OS observed in OSA to induce OS in the central nervous system (CNS). One mechanism implicates signaling from the carotid bodies. The carotid bodies are exposed to circulating markers and have direct efferent projections to the solitary tract nucleus (NTS). OS within the carotid bodies could elevate inflammatory signals and escalate OS in the NTS (37, 106). The NTS projects to several other forebrain regions, such as the hippocampus, entorhinal cortex (ETC), amygdala, and prefrontal cortex, to modify behavior and regulate homeostatic balance. OS signals could travel down these neuronal projections to distal regions and elevate OS there (33, 34).

The other mechanism proposed involves direct sensing of circulating factors by CNS components. Circulating OS directly impacts hypothalamic nuclei, such as the MnPO, which are accessible through the lateral ventricle (28, 74). The evidence of neural activation in autonomic regions such as the RVLM and NTS following CIH exposure supports this idea.

In previous studies, an elevation of NOX2 mRNA and protein expression occurred concurrently with a decrease in SOD2 in the NTS and RVLM following 10 days of CIH (106,

127). These were correlated to OS in the carotid bodies. Ablation of the carotid bodies eliminated this response. Interestingly, we saw no elevation of inflammatory signaling in the NTS nor a difference in protein expression in this region of animals experiencing CIH. This casts doubt on the hypothesis that early CIH exposure impacts brain OS levels through carotid body signaling. It is possible this effect may occur at a later time point and this likelihood warrants further investigation.

Neurons within the MnPO project to the rostral ventrolateral medulla (RVLM) which is involved in maintaining sympathetic pacemaker control of the autonomic nervous system. Because neurons in the RVLM exhibit elevated OS and inflammation due to systemic LPS stimulation and show evidence of activation during CIH, it has been assumed OS due to CIH would impact the RVLM in the same manner (146). Peng, et al., and Semenza, et al., (106, 127) have published data showing that 10 days of mild CIH stimulus in rats was accompanied by elevated expression of NOX2, a major contributor to OS, and decreased expression of SOD2, an antioxidant, in the RVLM as well as elevation of malondialdehyde (MDA), which is a downstream marker of lipid oxidation. In the study presented here, male rats subjected to 7 days mild CIH, did not exhibit elevations in OS, as measured by nitrotyrosine (NT). Conversely, a trend of decreasing NT was observed in the RVLM (figure 11). MDA is generated by ROS, while NT is RNS dependent (93). The decrease of NT may be reflective of an initial lack of NO consistent with ANG II signaling which first decreases nNOS, reducing NO production (49, 155), and at later time points induces iNOS transcription which greatly elevates it. It is possible the rise in NO occurs at a time point beyond 7 days. An elevation in ROS may occur more rapidly than the elevation in RNS due to the delay in iNOS transcription. Future studies should

compare the rate of NOX2 and iNOS transcription with generation of MDA and NT both for a more complete picture of the mechanisms involved.

It may also be possible the variation in protocols contribute to the differential effects seen here. CIH in these experiments provided equal amounts of hypoxic (10%) and normoxic (21%) O₂ exposure (90 seconds each), while the previously published studies utilized 5% O₂ for 15 seconds followed by 21% O₂ for 5 minutes (106, 127). The equal exposure times may be more protective in the RVLM nuclei than disproportionate timing. This is not unanticipated for the RVLM. Almodo, et al., demonstrated that, under mild CIH conditions (AHI=5.8), the presympathetic pacemaker neurons of the RVLM showed no changes in electrophysical properties although systemic symptoms of CIH were present (2). This suggests a much more extreme insult may be needed to alter homeostatic set points. This also agrees with prior research demonstrating the nature of OS and inflammatory signaling in neurons is specific to the type of stimulus providing the originating signal. Systemic LPS signaling did not produce effects in regions outside periventricular areas, whereas glutamate excitation and lesions to the hippocampus did elicit responses in mid and forebrain structures (138, 143).

Because the impact of early CIH exposure on ND was of concern in this project, punches from the SN, ETC, and hippocampus were also inspected. Along with the trend of NT decrease observed in the RVLM, a significant decrease of NT occurred in the hippocampus following CIH (figure 11). This observation may reflect the time course of apoptosis in the hippocampus associated with CIH exposure observed by Gozal, et al. (2003) (53). Apoptotic signals increased significantly following one day of severe CIH exposure, but returned to control levels by day 7. No elevation of OS was seen within SN or ETC nuclei in this study. The impact CIH has in these

regions associated with ND may occur at a time point other than seven days, similar to observations in the hippocampus.

The other hallmark of interest, elevated inflammation, is also a comorbidity of OSA (7, 23, 82, 90, 108, 115, 116). Uncontrolled, OS increases inflammatory signals which trigger both ROS and inflammatory pathways that lead to apoptosis (93). Similar to OS, IL-1 β expression is observed to elevate as aging occurs (31, 131, 134, 135, 152) and is more concentrated in ND pathological post-mortem tissue compared to healthy age-matched controls. IL-1 β and TNF- α have been documented in the CSF of patients with AD as well as in post-mortem hippocampal and frontal cortex tissue (16, 56, 115, 116). IL-1 β contributes to formation of characteristic plaques of AD pathology (56). Similar findings in the post-mortem CSF and nigro-striatal regions of patients with PD have been documented (16, 116), as well as the presence of cytokines within Lewy bodies characteristic of PD (123).

In addition to measuring OS levels, a panel of 10 cytokines with both pro- and anti-inflammatory properties were assessed as well (figure 1). The levels of all cytokines in circulation were significantly elevated in rats exposed to 7 days of mild CIH, except TNF- α (table 2). Their expression, including TNF- α , was significantly correlated to OS, except for KC/Gro (table 3). Even more significant was their correlation to IL-1 β levels (table 4). Moreover, inhibition of the AT1aR in the MnPO completely abolished the inflammatory escalation in the CIH exposed rats. It appears that OS caused by CIH through activation of neuronal AT1aR in the MnPO, significantly elevates inflammation by secretion of IL-1 β .

This phenomenon is comparable to other studies showing an elevation in OS and inflammation in conjunction with both hypertension and CIH (34, 86, 93, 146). Treatment of

either condition alleviates the associated OS, which diminishes resultant inflammation (3, 85, 86, 100, 139). This supports efforts to diagnose and treat cases of OSA early to prevent development of comorbidly associated diseases.

In brainstem regions, no difference in cytokine expression in the NTS and a significant decrease in IL-10, IL-4, IL-6, and TNF- α in the RVLM of rats exposed to 7 days CIH was observed (table 6). IL-10 and IL-4 are capable of attenuating pro-inflammatory signaling (118, 154). IL-6 and TNF- α classically lead to further inflammation and apoptosis (16, 114). All other cytokines measured in the RVLM were not significantly different from the normoxic controls.

This pattern of cytokine expression matches what was observed with OS markers in the NTS and RVLM in these experiments (figure 11, table 6). The inflammatory patterns seen here are different from LPS stimulation. Systemic LPS *i.v.* increases expression of IL-1 β , IL-6, and TNF- α in the RVLM (146). To date, no studies have been published regarding inflammation in the RVLM following CIH stimulus.

These patterns showed no correlation to plasma OS or inflammation, suggesting alternative mechanisms exist in CIH which are also exerting effects. What these mechanisms may be remains to be investigated. It appears 7 days of this model of mild CIH may serve a protective role in brainstem regions, in which OS and inflammation is repressed. Future studies addressing the role of inflammation in the RVLM at different time points and CIH severities would be beneficial.

Additionally, while inflammation is implicated in PD pathology, this study is the first to address the inflammatory state of the SN following CIH exposure. In this experiment, a significant elevation of activated microglial cells, as measured by CD11b expression, was

accompanied by a positive association between KC/Gro and TNF- α levels with plasma OS (figure 12). KC/Gro in rodents is similar to IL-8 in humans, serving to recruit monocytes and neutrophils and inducing membrane permeability (6). Unlike observations in the brainstem regions, circulating OS induced by CIH is associated with inflammation in the SN. This may prove to be a major contributor to the risk of developing PD by men with OSA and warrants further investigation.

Within the hippocampus and ETC, inflammatory processes have been well documented. Elevated IL-1 β and TNF- α expression has been observed in the hippocampus and surrounding regions following systemic LPS exposure, lesions to the hippocampus, and CIH exposures (15, 51, 76). Interestingly, the temporal patterns of expression and the neural cell types associated with expression vary depending on the stimulus. LPS *i.v.* generates inflammatory signaling by the microglial population first (76), whereas lesions or elevated glutamate signaling produce the IL-1 β signal in neurons between days 1-3 post-lesion, and the glial response is not observed until about 9 days later (15). This provides further evidence the type of insult inflicted dictates varying responses in brain regions.

Evidence of an inflammatory response, consistent with the progression of AD (70), was measured in the ETC. A trend of elevated KC/Gro, the molecule responsible for recruiting inflammatory cells (6), was observed. Similar to the SN, this trend was accompanied by a positive association with plasma OS (figure 7). OS due to CIH is also associated with inflammation in the ETC. This supports further investigation into mechanisms by which CIH elevates OS and inflammation in the ETC and how it may contribute to memory impairment.

Temporal studies of CIH on hippocampal regions have illustrated that IL-1 β is elevated dramatically following day 1 of CIH, but returns to normal levels by day 7 (53). This pattern of expression is accompanied by a significant deficit in working memory function. The experiments described here concur with those conclusions, showing no elevation of inflammation at day 7 due to CIH. No significant association with any plasma markers was observed at 7 days. The decreased NT observed as an indicator of OS, combined with the lack of elevated cytokine signaling, combined with other studies, suggests the impact CIH has on the hippocampus and the associated cognitive functions occurs at a different time point than 7 days.

Outlined here has been a case for the potential of the chronic intermittent hypoxia experienced by men with sleep apnea to contribute to oxidative stress and inflammatory profiles consistent with the progression of neurodegenerative diseases. CIH elevates circulating OS and inflammation. One of the molecular mechanisms through which this is accomplished is elevated ANG II activating the AT1a receptor in the MnPO. Blocking this receptor blocks the downstream systemic OS and inflammatory effects. The elevation in OS contributes to inflammation in the SN and the ETC, both of which are implicated in the initial stages of Parkinson's and Alzheimer's disease respectively (figure 13). Circulating OS may prove to be a useful biomarker of the risk of neurodegeneration in either the ETC or SN. Which pathway is more affected may depend on genetic and environmental factors. While further research into these mechanisms is necessary, it may currently be advisable for clinicians to monitor male patients with any severity of OSA for signs of cognitive or motor function impairment early on to enable early effective intervention and prevent neurodegeneration.

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