

Sleep Apnea Impairs Hippocampal Function and Adult Neurogenesis

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Abstract

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Sleep apnea is a respiratory disorder characterized by periods of sleep during which no effective breaths are taken. Recurrent episodes of apnea caused by upper airway obstruction and/or cessation of central respiratory signaling results in repeated bouts of hypoxemia. Sleep apnea is quite common, affecting approximately 10% of adults in western nations, but a large proportion of patients remain undiagnosed and untreated. Continuous positive air pressure devices, or CPAP therapy, are effective therapeutics to maintain upper airway patency and prevent apneas, but are not universally available nor tolerated by sleep apnea patients. Obesity, the single biggest risk factor for the development of sleep apnea, is even more common and its prevalence is expected to rise. Therefore, sleep apnea represents a considerable health burden for the world population.

Patients with sleep apnea have a greater risk of developing cardiovascular complications, such as hypertension and stroke, and as a result, much research has focused

on the mechanisms of damage to the cardiovascular system. However, OSA is also known to impact the central nervous system. Some proportion of sleep apneas are generated by a failure of the central respiratory network to signal properly. Furthermore, patients with untreated sleep apnea experience mild cognitive impairments and are reported to have alterations in the activity of a number of brain regions. Even so, the mechanisms by which sleep apnea affects the central nervous system remain largely unknown.

As both a critical regulator of learning and memory and one of only two structures in the mammalian brain capable of adult neurogenesis, the hippocampus may be particularly at risk in untreated sleep apnea. Indeed, individuals with sleep apnea experience mild cognitive impairment associated with changes to the hippocampus. Although oxygen homeostasis is a well-recognized factor that can influence multiple hippocampal processes, the impact of intermittent hypoxia (IH), a principal consequence of sleep apnea, on hippocampal neurophysiology remains unclear. I hypothesized that intermittent hypoxia would cause dysfunction in multiple stages of adult neural development to impair circuit function of the dentate gyrus (DG), thus contributing to injury of the hippocampus.

The studies related in this dissertation utilize behavioral, electrophysiological, and immunohistological techniques to describe the effects of chronic intermittent hypoxia (IH) exposure on the neurophysiology of the murine hippocampus. IH impaired spatial memory in the Barnes maze apparatus and correlated with attenuated long-term potentiation (LTP) in the DG. Immunohistological analyses revealed that IH differentially perturbs adult neurogenesis by decreasing the number of new-born neurons, while simultaneously increasing neuroprogenitor cell proliferation. Although administration of the superoxide anion scavenger antioxidant, MnTMPyP, mitigated LTP suppression and prevented adult

born neuron loss, IH-dependent proliferation of neuroprogenitor cells was unaffected. These data demonstrate that IH disrupts multiple processes in the DG that are both dependent on, and independent of, reactive oxygen species (ROS). These novel findings identify IH-induced changes in cellular and functional correlates of hippocampal learning and memory that likely contribute to cognitive deficits in sleep apnea. Further work is required to determine the non-ROS-mediated mechanisms that affect the neural progenitor pool of the hippocampus and whether either mechanism could serve as a future target for sleep apnea therapeutics.

Dedicated to:

*My Great-grandfather, Reuben Bernstein,
for never wavering in his belief that I could become a doctor
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Chapter 1

Introduction

Obstructive Sleep Apnea (OSA) is a highly-prevalent type of sleep-disordered breathing defined by upper respiratory obstruction that leads to recurrent episodes of hypoxia (Burwell et al., 1956; Quan et al., 1997). OSA affects approximately 10% of the U.S. adult population, and is anticipated to rise along with its highest risk factor, obesity (Young et al., 2009; Bhattacharjee et al., 2012). Since one third of Americans are considered obese, OSA represents a considerable health burden for the American population (Watson, 2016). Patients with this disorder have a higher risk of developing cardiovascular, metabolic, psychological and neurophysiological complications (Somers et al., 2008). Patients self-report daytime somnolence, and demonstrate mild cognitive deficits when OSA is left untreated (Dewan et al., 2015). Cognitive deficits indicate a potential loss of active neurons within one or more brain circuits located throughout the cerebrum. In the few brain regions capable of adult neurogenesis, neuronal loss could be attributed to injury or a failure to generate newly-born neurons in sufficient number for optimal circuit function (Wang et al., 2012). The experiments described in this dissertation examine the neurophysiological effects of intermittent hypoxia, a principle consequence of OSA, on adult hippocampal neuron development, activity, and function.

1.1 Definition and History of Sleep Apnea

Sleep is essential for healthy neurological development and function. We spend approximately one third of our lives sleeping, yet a majority of surveyed adults report difficulties in initiating or maintaining sleep at least a few nights a week (The National Sleep Foundation, 2014). In 2015, approximately half of American adults surveyed also reported that daytime somnolence interfered with their normal activities at least a few days a month (The National Sleep Foundation, 2015). The long-held notion of sleep as a passive and uniform state has been upended as research demonstrates the complex cyclical periods of electrical activity, memory consolidation, and behavior (Alhola and Polo-Kantola, 2007; Pelayo and Dement, 2011). Although it is commonly accepted that sleep is important, it is not often given a high level of priority in adult life (Barnes and Drake, 2015). Parents of young children report reduced number of hours of sleep and great daytime somnolence, which was more severe if the parent was also working (Gay et al., 2004; Sinai and Tikotzky, 2012). Insufficient sleep leads to reductions in memory, executive function, and attentional capacity that can negatively affect mood, emotion, and even interpersonal relationships (Alhola and Polo-Kantola, 2007). Sleep disorders are highly-prevalent in western adult populations (Peppard et al., 2013), and presentations are expected to increase as the population ages. While often accepted as normal by clinicians and members of the general public alike, sleep disorders among older adults are often a result of, and contributor to, a variety of medical and psychological comorbidities (Bloom et al., 2009).

Sleeping troubles in patients can be due to many different causes. A primary care physician could diagnose anything from insomnia, circadian rhythm sleep disturbances,

and restless legs syndrome to narcolepsy, REM sleep behavior disorders, or sleep disordered breathing (SDB) (Panossian and Avidan, 2009). SDB is a major contributor to sleep problems, consisting of distinct and overlapping syndromes of hypoventilation, central sleep apnea, and obstructive sleep apnea (OSA) (Panossian and Daley, 2013). All disorders under the SDB umbrella are primarily characterized by pauses in airflow during sleep. Defined by obstruction in the upper airway, OSA results in periods of sleep during which no effective breaths are taken and periodic hypoxemia is recorded (Burwell et al., 1956; Quan et al., 1997).

The earliest clinical descriptions of OSA occurred in 1965 simultaneously by Gastaut and Jung (Gastaut et al., 1965; Jung and Kuhlo, 1965). Both groups report obese patients with hypoventilatory responses during sleep that result in daytime somnolence, hypoxemia, and bradycardia. However, several earlier reports deserve mention. Back in 1836, Charles Dickens described the physical characteristics that would come to be associated with OSA in the depiction of his character Joe, the fat boy, in *The Posthumous Papers of the Pickwick Club* (Dickens, 1836). As the nickname suggests, Joe is overweight, but also constantly sleepy; he often does not respond to his name being called because he has fallen asleep, which is recognized by his companions by both his inattention and snoring. When a business executive presented at Peter Bent Bingham Hospital in 1956 with obesity, fatigue and somnolence, Burwell and colleagues were so struck by the similarities to Dickens's Joe that they named his physical manifestations a "Pickwickian syndrome" (Burwell et al., 1956). Burwell determined that his patient had low arterial oxygen saturation and elevated arterial carbon dioxide tension, which were partially alleviated by enforced weight reduction. This study failed to acquire data specifically during sleep and

associated the somnolence specifically with hypercapnia, thus missing the opportunity to discover sleep apnea. In 1960, a group from Heidelberg University Hospital in Germany described a patient who complained of recurring morning headaches (Gerardy et al., 1960). By polygraphy taken during a nap, they demonstrated pauses in sleep respiration associated with loud recovery snores, but did not define this as sleep apnea. A similar report was published by Drachman and Gumnit in 1962, when an obese and somnolent patient at the National Institutes of Health (NIH) in the USA was evaluated by electroencephalography (Drachman and Gumnit, 1962). They identified repetitive pauses in air exchange despite persistent movements of the abdominal and thoracic cavities and also enforced strict weight reduction to resolve the daytime somnolence. These data were best integrated in “the sleep apnea syndromes” by Guilleminault in 1976 (Guilleminault et al., 1976). This seminal text defined apneas by length of air flow disruption, the syndrome by number of apneas per hour of sleep, and described three distinct forms of the syndrome. Beginning with common clinical symptoms, Guilleminault and colleagues systematically describe the pathological changes found in sleep apnea patients, with detailed focus on both respiration and cardiac function. Since then, multiple definitions of obstructive sleep apnea were used within the field until the American Academy of Sleep Medicine Task Force standardized the definition in 1999 along with European Respiratory Society, Australasian Sleep Association, and American Thoracic Society (Anon, 1999). The accepted definition of obstructive sleep apnea is the complete (apnea) or partial (hypopnea) obstruction of the upper airways during sleep.

1.2 Diagnosis and Epidemiology of Obstructive Sleep Apnea

Obstructive sleep apnea is highly-prevalent in the general population. Three large cohort studies of American's sleep quality completed in the late 1980s and early 1990s reported the prevalence of sleep-disordered breathing to be about 8% in women and 24% in men, although the ranges were quite broad (Young et al., 1993; Quan et al., 1997; Bixler et al., 2001). In 2013, the rates were revised based on more recent epidemiological data of known risk factors to result in a 26% prevalence among American adults (Peppard et al., 2013). Other recent studies completed on non-American cohorts in France, Saudi Arabia and Switzerland produced qualitatively similar results (Fuhrman et al., 2012; Heinzer et al., 2015; Wali et al., 2017). A recent review of the literature that excluded repeat studies, small cohorts, and out-of-date methodologies reports that OSA prevalence ranged from 9-38% of the general population (Senaratna et al., 2017). If over a third of adults are potentially suffering from OSA, screening for common OSA symptoms must become routine during visits to primary care physicians.

There are three cardinal symptoms of OSA that clinicians consider when evaluating patients in the primary care setting: loud snoring, daytime somnolence, and recurrent episodes of apneas during sleep (Epstein et al., 2009). As most people are not fully aware of their behavior when they themselves are asleep, a discussion with the patient's significant other or bed partner is often helpful. In fact, a mnemonic commonly-used by clinicians is the three S's: snoring, sleepiness, and significant other report. Definitive diagnoses typically require polysomnography to be completed overnight to detect the frequency of apneic and hypopneic events. The average number of events per hour of sleep is known as the apnea-hypopnea index (AHI) and is used to determine presence and severity of OSA. An AHI greater than 15 is definitively indicative of OSA, while an AHI between 5 and 15 requires

the presence of associated symptoms, such as daytime somnolence or fatigue, to earn the same title (Park et al., 2011). An AHI greater than 30 is considered to be a severe representation of OSA. Overnight sleep studies to exclude OSA diagnosis are relatively inconvenient for patients, and are therefore only recommended to patients exhibiting cardinal OSA symptoms along with known risk factors.

OSA prevalence in the general population has proved difficult to definitively tie down, although studies in smaller, sub-populations have been informative (Fuhrman et al., 2012; Kleisiaris et al., 2014; Acker et al., 2017; Alonderis et al., 2017; Hein et al., 2017; Senaratna et al., 2017). There are several groups that are known to have higher rates of OSA, and their prevalence within the general population must be taken into account when making predictions across an entire population (Peppard et al., 2013). The most widely accepted non-diagnostic feature of OSA is excessive weight, as noted by the earliest Pickwickian syndrome reports. Indeed, obesity is a significant risk factor for OSA that clinicians should be aware of. During the Wisconsin Sleep Cohort Study, researchers identified a 4-fold increase in OSA prevalence for every standard deviation increase in body mass index (BMI) (Young et al., 1993). Since then, many groups have demonstrated increases in OSA prevalence in accordance with the severity of obesity (Bixler et al., 1998, 2001; Peppard, 2000; Durán et al., 2001). Using data from the Wisconsin Sleep Cohort Study and national epidemiological information, Peppard and colleagues estimated the prevalence of SDB in the adult US population based on sex, age, and BMI (Peppard et al., 2013). They report a 1.4% and 7% prevalence of SDB in young women and men, respectively, with normal/underweight BMIs of less than 25; the rates nearly double for every increase in BMI of ≥ 5 , corresponding with increasing level of obesity severity. Nearly

80% of males under the age of 50 with a BMI \geq 40 are predicted to suffer from SDB. Change in weight over time is directly correlated with OSA severity (Peppard, 2000; Tishler et al., 2003; Newman et al., 2005). Following participants of the Sleep Heart Health Study, Newman and colleagues demonstrated that weight gains of >10kg in men, or approximately 22 pounds, result in 5.2-fold increase in the odds of OSA severity escalation. This data confirms earlier reports from the Cleveland Family Study demonstrating increased AHI score along with increased BMI, and data from the Wisconsin Sleep Cohort Study, which showed a 10% gain in weight predicted a 32% increase in OSA severity (Peppard, 2000; Tishler et al., 2003). With a hopeful eye toward the reduction of OSA prevalence and severity, these reports have also demonstrated that opposite changes in BMI can reduce OSA severity. Enforced weight loss in the earliest of OSA presentations, AKA Pickwickian syndrome, showed the potential for a reduction in the severity of symptoms and has been the recommended treatment of OSA since at least 1985 (Burwell et al., 1956; Drachman and Gumnit, 1962; Smith et al., 1985). Newman and colleagues demonstrated that weight loss slowed the OSA progression in the Sleep Heart Health Study, while a more recent small study by the Kuopio Sleep Apnea Group in Finland documents an 80% reduction in the progression of OSA in a successful weight loss group as compared to an unsuccessful group (Newman et al., 2005; Tuomilehto et al., 2014). Tuomilehto and colleagues in Finland focused on an important aspect of the clinical setting: failure to induce and maintain weight loss. Despite known health complications related to obesity, the rate of US adult obesity has held steady at 35% from 2003-2012, suggesting that more invasive techniques may be necessary for a reduction in OSA prevalence (Ogden et al., 2014). Grunstein and colleagues demonstrated that bariatric-surgery induced weight loss

of approximately 23% resulted in a 15% reduction in the frequency of apneas that was not observed in non-surgical controls (Grunstein et al., 2007). This data is consistent with earlier reports from several small cohort studies (Fritscher et al., 2007). A more recent meta-analysis of common OSA treatments also demonstrated that weight loss reduces the severity of OSA (Iftikhar et al., 2017). Taken together, these studies demonstrate the role that weight plays on the prevalence and severity of OSA. Interestingly, other size-related measurements have been poor predictors of OSA prevalence and severity (Punjabi, 2008). Researchers from the Sleep Health Heart Study report that increased neck and waist circumference, measurements of relative fat distribution, are independently associated with moderate to severe OSA, although the use of these factors separate from BMI remains controversial (Young et al., 2002).

Another long-ago recognized distinction in the development of OSA was an increased susceptibility in males. Clinical studies have shown that between 5 and 8 times as many men are referred to sleep studies than women (Wilhoit and Suratt, 1987; Redline et al., 1994). Follow-up epidemiological studies suggest that the ratio of men to women is closer to 2 or 3:1, but confirm a disparity nonetheless (Young et al., 1993; Bixler et al., 2001; Durán et al., 2001; Wahner-Roedler et al., 2007; Basoglu and Tasbakan, 2017; Foster et al., 2017; Senaratna et al., 2017). Several explanations are possible for this disparity. First, many of the characteristics of OSA as a syndrome were defined in male patients, and female patients may present with slightly different symptoms. Indeed, it has been reported that women with OSA are more likely to describe fatigue and lack of energy as their primary symptom than men (Chervin, 2000; Shepertycky et al., 2005; Wahner-Roedler et al., 2007). Second, since clinical presentation is often dependent upon the report of

witnessed apneas by a significant other or bed partner, there may be a differential in recognition by the significant other of the female patient. In one study, female bed partners of OSA patients described lower sleep and life quality for themselves than male bed partners of OSA patients, suggesting that the female bed partners are more sensitive to their partners' sleeping habits than male bed partners (Breugelmans et al., 2004). Thirdly, lower female prevalence of OSA may be due to a failure of clinicians to recognize common symptoms in females, since it is known that OSA is more common in males, a sort of self-fulfilling diagnosis (Lindberg et al., 2017). In any case, the under recognition of OSA is important for public health as delayed diagnosis and treatment in women can contribute to increased morbidity and treatment costs (Banno et al., 2006; Knauert et al., 2015).

An additional explanation for the disparity in OSA prevalence is hormonal influences. The potential role that testosterone plays in increasing OSA has been recognized for at least 30 years, supported by studies which reported that AHI increased in hypogonadal men after testosterone administration (Matsumoto et al., 1985). More recent clinical trials demonstrated that testosterone administration exacerbated OSA in older men (Liu et al., 2003; Hoyos et al., 2012). Similarly, women with polycystic ovarian syndrome, characterized by increased testosterone levels, are more likely to OSA than reproductively healthy women (Fogel et al., 2001; Vgontzas et al., 2001; Yang et al., 2009; Mokhlesi et al., 2012). In fact, OSA severity in these women is directly correlated with serum and unbound testosterone levels (Fogel et al., 2001). Complementary, reproductively healthy women with moderate to severe OSA are reported to have lower levels of the female sex hormones estradiol and/or progesterone (Netzer et al., 2003). Disease prevalence is also greater in post-menopausal women that have reduced estrogen levels (Bixler et al., 2001).

Furthermore, hormone replacement therapy of estrogen/progesterone has been associated with lower OSA prevalence in post-menopausal women (Bixler et al., 2001; Shahar et al., 2003). The influence of sex hormones may also affect the presentation of different secondary sexual characteristics in the anatomy of the upper airway of men, thus inhibiting airflow to induce apneas or hypopneas (Jordan and McEvoy, 2003).

In addition to sex-related differences in prevalence, sleeping and breathing patterns differ between male and female OSA patients. After controlling for variances in BMI, men display increased OSA severity, as demonstrated with greater AHI levels (O'Connor et al., 2000; Ware et al., 2000; Walker et al., 2001; Youn et al., 2015; Leppänen et al., 2017). Contributing to this difference is that AHI is greater in men, generally in the supine position, during non-rapid eye movement (non-REM) despite similar AHI in REM sleep (O'Connor et al., 2000; Gillman et al., 2012; Youn et al., 2015). Tashkandi and colleagues report that AHI during non-REM is elevated in men primarily due to an increase in apneic events without a change in hypopneic events (Tashkandi et al., 2005). Complementary, disruptive sleep events tend to be shorter, easier to recognize during REM sleep, and result in less oxyhemoglobin desaturation in women (O'Connor et al., 2000; Ware et al., 2000; Koo et al., 2008). Interestingly, the differences in OSA prevalence and severity between the two sexes is greatest at relatively younger ages, becoming indistinguishable after the age of 50 (Tishler et al., 2003; Koo et al., 2008; Youn et al., 2015).

Sleep-related difficulties become increasingly common with advancing age (Vaz Fragoso and Gill, 2007). Common complaints consist of difficulty falling asleep, difficulty maintaining sleep, and reduced total amount of night-time sleep obtained (Ford and Kamerow, 1989; Gislason et al., 1993; Foley et al., 1995; Vaz Fragoso and Gill, 2007).

Epidemiological studies reveal that more than half of senior aged adults have some form of chronic sleep-related complaints (Foley et al., 1995). As such, the prevalence of OSA in older populations is consistently greater than that in younger populations, and the risk of developing OSA or increasing the severity of OSA increases over time (Senaratna et al., 2017). Newman and colleagues demonstrated that, even in the absence of weight gain, between 10 and 20% of Sleep Heart Health Study participants developed moderate to severe OSA during the 5-year period of observation (Newman et al., 2005). The group attributed these changes primarily to the increase in age, as weight gain was controlled for. A small cohort study conducted in San Diego in the early 1990s reported that, of patients 65-99 years old, 70% of men and 56% of women displayed moderate to severe OSA (Ancoli-Israel et al., 1991). Follow-up studies later in the decade also show OSA prevalence to increase with age, starting as low as 3.2% for a 20-44-year-old age group and increasing stepwise to 18.1% for a 61-100 year old age group (Bixler et al., 1998). More recent meta-analysis of world-wide general population OSA prevalence confirms both the increased prevalence and increased severity reported with advancing age (Senaratna et al., 2017). Heinzer and colleagues report that the advanced age groups of the HypnoLaus Study in Switzerland displayed prevalence as high as 84% overall and 90% in aged men (Heinzer et al., 2015). Moderate to severe OSA is approximately twice as prevalent in aged versus young populations, at 36% and 17% respectively (Tufik et al., 2010; Lee et al., 2014; Heinzer et al., 2015). Interestingly, while OSA severity increases along with age, severe OSA is most prevalent in middle aged men and elderly women (Bixler et al., 2001; Li et al., 2015). Of post-menopausal women, OSA prevalence is the greatest in those that are not on hormone replacement therapy, suggesting that reduced estrogen and/or progesterone is a

potential contributor to this sex discrepancy in aged women (Bixler et al., 2001). An additional caveat to this data on OSA and age is that it may not be able to correct for the impact of co-morbidities with age. Recent evidence supports independent association between intermittent hypoxia, a cardinal symptom of OSA, and diseases common with increased age: diabetes mellitus, dyslipidemia, metabolic syndrome, and hypertension (Trzepizur et al., 2013; Tkacova et al., 2014; Heinzer et al., 2015; Torrella et al., 2015). For these reasons, advanced age should be considered a considerable risk factor for OSA.

Until recently, most of the population-based studies on the prevalence of OSA were focused on populations in North America, Europe, and Australia. The last decade has seen these studies extended to China, India, Korea, Brazil, and Saudi Arabia (Ip et al., 2001, 2004; Kim et al., 2004; Udwadia et al., 2004; Tufik et al., 2010; Lee et al., 2014; Wali et al., 2017). These studies demonstrate that the prevalence of OSA in Asian, Middle Eastern, and South American communities is comparable to North American and European communities. Interestingly, while obesity is less prevalent in Asian populations, OSA prevalence is just as great as in the West. Within the United States, a few studies have attempted to breakdown OSA prevalence amongst various racial sub-groups. OSA prevalence amongst middle-aged African-Americans has been reported to be comparable to that of other racial groups, but African American young adults and seniors have increased prevalence, after controlling for BMI (Redline et al., 1994; Ancoli-Israel et al., 1995; Young et al., 2002). A more recent, larger cohort study reports an increase in sleeping disturbances across all age groups of African Americans, and a particularly increased risk of OSA in African-American women (Fülöp et al., 2012). There are also caveats to consider with regard to this data: in the US, much of the African American population is in a lower socioeconomic bracket, has reduced

access to medical care, and has slightly greater prevalence of obesity. Follow-up studies are needed to determine whether race may be a surrogate for other predisposing features and whether the additional risk would disappear if confounding factors were addressed.

In addition to weight, sex and race, there are mechanical properties of the upper airway that could increase its propensity to collapse during sleeps, thus affecting the prevalence of OSA. Several common imaging modalities have been used to demonstrate skeletal and soft-tissue structural abnormalities in patients with OSA. Features such as tonsillar hypertrophy, enlarged tongue or soft palate, overbite, inferiorly positioned hyoid bone, and decreased posterior airspace can narrow the dimensions of the upper airway to promote the occurrence of apneas and hypopneas (Cistulli, 1996). Even without obvious clinically relevant craniofacial abnormalities, subtle differences in maxillary or mandibular size could increase susceptibility to OSA. In fact, a meta-analysis of studies on OSA and craniofacial risk factors indicated that the length of the mandible is an important measure associated with increased OSA risk (Miles et al., 1996). In a series of small cohort studies, children born with craniofacial microsomia have been reported to have between 7 and 67% OSA prevalence, supporting the idea that skeletal structural abnormalities affect OSA risk (Caron et al., 2015). Recent studies suggest that tooth loss may increase OSA prevalence and that differences in craniofacial features between Asian and European populations may explain why OSA is equally prevalent despite decreased obesity prevalence in Asian populations (Li et al., 2000; Lam et al., 2005; Sanders et al., 2016). Collectively, these studies support the idea that craniofacial abnormalities are important in the development of OSA. Clinicians should take craniofacial differences into account along

with obesity, sex, and age when determining risk for OSA and deciding whether to refer patients to overnight polysomnography for diagnosis.

1.3 Sequelae of Obstructive Sleep Apnea

Periodic breathing cessation during OSA results in a multitude of physiological responses (Dempsey et al., 2010). The most common are recognized as presenting symptoms to clinicians: snoring, daytime sleepiness, obesity, and poor sleep. OSA patients suffer from disturbances in both REM and NREM sleep (Guilleminault et al., 1976; Carreras et al., 2014; Gabryelska et al., 2017). However, OSA also contributes to a wide range of serious medical problems (Marshall et al., 2014). In its severe form, OSA increases risk for premature mortality, cardiovascular disease, and neurological disorders (Marshall et al., 2008; Punjabi, 2008). OSA has also been shown to increase the severity of pre-existing conditions (Gottlieb et al., 2010). The effects of sleep apnea on the body can be divided into two separate, yet interrelated, categories discussed below: cardiovascular and neurological manifestations.

1.3.1 Cardiovascular and Metabolic Manifestations

Large epidemiological studies of SDB and OSA repeatedly demonstrate that untreated OSA is an independent cardiovascular risk factor (Shahar et al., 2001; Marin et al., 2005; Young et al., 2008). Compared to the general population, the prevalence of OSA is greater for people with cardiovascular conditions, such as hypertension (Fletcher et al., 1985; Logan et al., 2001; Mokhlesi et al., 2014; Muxfeldt et al., 2014), ischemic heart disease (Peker et al., 1999; Mooe et al., 2001; Won et al., 2013; Zhao et al., 2014), heart

failure (Ferrier et al., 2005; Javaheri, 2006; Geib et al., 2015; Arzt et al., 2016), and stroke (Bassetti and Aldrich, 1999; Kaneko et al., 2003; Brown et al., 2014; Chang et al., 2014; De Lott et al., 2014). Severe OSA can contribute to the emergence of these same co-morbidities as well (Shahar et al., 2003; Chang et al., 2014; Brown et al., 2015).

Effort has been made to relate the mechanism of OSA to the associated increased cardiovascular disease risk. Unfortunately, OSA is not a simple disease but rather heterogeneous, characterized by multiple mechanisms such as intermittent hypoxemia, intermittent hypercapnia, decreases in intrathoracic pressure, and repeated arousals from sleep. Intermittent hypoxia caused by OSA is believed to be the major contributor to cardiovascular disease onset, with changes to intrathoracic pressure and sleep fragmentation playing smaller roles in disease progression (Kumar et al., 2006; Baguet et al., 2009; Johansson et al., 2011; Linz et al., 2011; Yamamoto et al., 2013; Gonzaga et al., 2015). The repeated periods of desaturation and re-saturation of hemoglobin partly explain the presence of hypertension since this pattern increases activity of the sympathetic nervous system, renin-angiotensin-aldosterone pathway, and peripheral vasoconstriction (Fletcher et al., 1992; Carlson et al., 1996; Kumar et al., 2006; Somers et al., 2008). In NREM sleep, healthy patients will experience decreased respiratory rate, blood pressure, and heart rate (Tzivoni and Stern, 1973). In congruence, respiratory rate, blood pressure and heart rate all rise during REM sleep (SNYDER et al., 1964). The relationship between sleep and cardiac arrhythmias is well defined, with clear sleep-wake patterns in both atrial and ventricular arrhythmias and state dependent heart rate alterations (GASSEL et al., 1964; Baust and Bohnert, 1969; Verrier et al., 1996). Approximately 40% of paroxysmal atrial fibrillations and 15% of fatal ventricular

arrhythmias occur during sleep (Brodsky et al., 1977; Lavery et al., 1997; Yamashita et al., 1998). OSA patients have a greater risk of sudden cardiac death and are more likely to die at night as compared to those without OSA who are more likely to die in the morning (Gami et al., 2005, 2013; Kuniyoshi et al., 2008). Using a multivariate analysis, the independent predictors of atrial fibrillation and sudden death identified were not AHI number, but rather the oxygen desaturation index, indicating that hypoxemia plays an important role in arrhythmogenesis and cardiac death in OSA (Gami et al., 2007; Ghias et al., 2009).

Decreased oxygen saturation during sleep is associated with increased likelihood of ST segment depression, a common EKG marker of cardiac ischemia, further supporting this idea (Moore et al., 2000). Animal studies have also attempted to identify the mechanism by which OSA contributes to cardiac morbidity. Brooks and colleagues demonstrated that obstructive apneas increased night time blood pressure and persisted into the daytime, while arousals from sleep also increased night time blood pressure, but without alteration to daytime blood pressure (Brooks et al., 1997). Tkacova and colleagues recently showed that nocturnal oxygen desaturation predicts prevalent hypertension in the European Sleep Apnoea Database study (Tkacova et al., 2014). These studies also suggest that nocturnal hypoxemia, and not arousals, is the primary contributor to OSA-related hypertension.

In addition to hypertension and cardiac arrhythmias, OSA affects cardiovascular pathologies through hormonal and metabolic dysfunction. Decreased circulating nitric oxide levels and increased endothelin-1 signaling in OSA result in systemic vasoconstriction (Carlson et al., 1996; Ip et al., 2000; Kato et al., 2000; Gjørup et al., 2007; Caimi et al., 2015). Multiple studies demonstrate that the carotid body also mediates vasoconstriction in OSA via increased adrenal catecholamine secretion (Semenza, 2000;

Peng et al., 2001; Elmasry et al., 2002; Souvannakitti et al., 2009). Catecholamine hormones increase blood pressure via vasoconstriction, but also by accelerating the uptake of low density lipoprotein into and reducing cholesterol efflux out of atherosclerotic plaques (Born, 1991; Xu et al., 2015). Dyslipidemia is highly prevalent among OSA patients, increasing the risk of vascular events due to the atherosclerotic plaque rupture (Quintero et al., 2013; Nadeem et al., 2014; Nagayoshi et al., 2016). OSA is also associated with hypercoagulability by increases in prothrombotic factors, fibrinogen, and platelet activation (von Känel et al., 2007; Somers et al., 2008; Tosur et al., 2014). All of these elements contribute mechanistically to an increased risk of myocardial infarction and stroke in OSA patients.

Separate from the cardiovascular complications of OSA, there are metabolic syndromes associated with OSA as well. Impaired glucose tolerance is common in OSA patients and is independently associated with AHI > 5 after adjusting for BMI (Ip et al., 2002; Punjabi et al., 2002). Exposing healthy patients to acute intermittent hypoxia decreases insulin secretion, insulin sensitivity, and glucose tolerance, laying the groundwork for developing metabolic syndrome (Oltmanns et al., 2004; Louis and Punjabi, 2009). Heart rate was also increased in the healthy volunteers, suggesting increased sympathetic drive. The Sleep Heart Health Study showed an independent association between OSA severity and the development of type 2 diabetes mellitus, after adjusting for abdominal girth (Punjabi et al., 2004). A more recent large cohort study from St Michaels Hospital in Toronto, Canada demonstrated a qualitatively similar association between OSA severity and diabetes onset over the course of 15 year data collection (Kendzierska et al., 2014). Nakata and colleagues recently demonstrated an association between the severity of

SDB and the glycemic variability observed in diabetes patients as well (Nakata et al., 2017). Multiple other studies show a similar correlation, but either do not correct for obesity levels or lose the correlation when corrected, indicating that obesity is an important confounding variable (Reichmuth et al., 2005). Obesity may be a shared risk factor for both OSA and cardiometabolic dysfunction, increasing the severity of either disorder when present. Additionally, OSA patients have been reported to have increased systemic markers of oxidative stress, which affects multiple metabolic pathways and could worsen a variety of co-morbidities (Drager et al., 2013; Caimi et al., 2015; Lavie, 2015).

1.3.2 Neurophysiological and Psychological Manifestations

Several consequences of OSA indicate that the nervous system is affected by the disease. The fact that OSA occurs only during sleep while the presence of common risk factors remain throughout both wakefulness and sleep indicates that the autonomic nervous system is negatively impacted during OSA. Indeed, the cardiovascular abnormalities observed during OSA and described above also implicate aberrant nervous activity as heart rate and the neuronal control of breathing are closely linked. Respiratory sinus arrhythmia, when heart rate is increased during inspiration and slowed during expiration, is observed in normal and augmented breathing patterns of healthy patients during both sleep and wakefulness (George and Kryger, 1985; Galletly and Larsen, 1998; Kabir et al., 2013; Sola-Soler et al., 2015). The coupling of respiration and heart rate is important for homeostatic regulation of blood gases and for critical nervous system functions, such as arousal (Hayano et al., 1996; Ben-Tal et al., 2012). Sighs, and associated respiratory sinus arrhythmia, commonly occur at the onset of arousal, and are believed to

play a role in the recovery of airway patency after an obstruction (Remmers et al., 1978; Roberts et al., 1986; Wulbrand et al., 2008). During healthy sleep, the cardiovascular system is in a state of relaxation, where metabolic rate, heart rate, and input from the sympathetic nervous system are all reduced (Somers et al., 1993). In OSA patients, intermittent hypoxemia causes repeated arousal responses that disrupt the normal cardiovascular relaxation during sleep and result in a persistent increase in sympathetic tone during wakefulness (Gastaut et al., 1966; Bradley and Floras, 2003a, 2003b, 2009; Somers et al., 2008). Arousal and increased sympathetic input is beneficial during an obstructive event as it prevents a sustained period of hypoxemia, but the type of chronic repeated arousals observed in OSA lead to an imbalance between the sympathetic and parasympathetic branches of the autonomic nervous system (Bradley and Floras, 2003a, 2003b). Such imbalances are also observed in other breathing disorders such as apneas of prematurity, Sudden Infant Death Syndrome, Familial Dysautonomia, and Rett Syndrome, as well as hypertension and heart failure (Kahn et al., 1988; Meny et al., 1994; Franco et al., 1998, 2003; Weese-Mayer et al., 2006; Triposkiadis et al., 2009; Kishi, 2012; Garcia et al., 2013; Carthy, 2014; Bhardwaj and Dunlap, 2015).

While it is generally agreed that central apneas and apneas of prematurity result from imbalances in autonomic signaling, OSA patients also suffer from failures of the autonomic nervous system. Inconsistent respiratory rhythms are commonly observed in patients with severe OSA (Ramirez et al., 2013). Intermittent hypoxia has been recently reported to increase irregular output from the preBötzinger complex, a brainstem network critical for breathing and inspiratory rhythm generation (Lieske et al., 2000; St-John et al., 2007; Tan et al., 2008; Ramirez, 2011; Schwarzacher et al., 2011; Koch et al., 2013). This

study also demonstrated intermittent hypoxia changes the input-output relationship between the preBötzinger complex and the hypoglossal motor nucleus, leading to periodic transmission failure of the hypoglossal nerve. Temporary cessation in hypoglossal activity triggers pharyngeal collapse to create an upper respiratory obstruction (Remmers et al., 1978). Several other factors contribute to the cessation of hypoglossal activity such as reduced sensory input, increased arousal threshold, and failures in motor neuron output (Horner, 2007; Prabhakar et al., 2007; Chamberlin, 2013). This and the presence of enteric nervous system irregularities indicates that OSA negatively impacts the nervous system (Anon, 1999).

In addition to alterations in the autonomic nervous system, OSA also impacts central nervous system function (Dewan et al., 2015). Patients with untreated OSA report a variety of daytime cognitive symptoms such as difficulty concentrating, changes in mood, memory difficulties and excessive daytime sleepiness (Barnes et al., 2004; Torelli et al., 2011; Olaithe and Bucks, 2013). Children with OSA demonstrate reduced academic performance when compared to healthy sleeping peers (Gatica et al., 2017). Analysis of memory, cognitive and psychomotor function in OSA patients has been heterogeneous, with a patchwork of different tests utilized and a mix of conclusions reached, but the majority coalesce around mild central nervous system impairment such as reduced executive function (Naegele et al., 1995; Saunamaki and Jehkonen, 2007; Canessa et al., 2011; Torelli et al., 2011; Gozal et al., 2012; Sforza and Roche, 2012; Csabi et al., 2014; Djonlagic et al., 2014). A recent meta-analysis by Leng and colleagues identified sleep disordered breathing as an independent risk factor for developing cognitive impairment and reduced executive function (Leng et al., 2017). The severity of sleep disturbance and hypoxemia in OSA is

positively associated with the level of impairment in executive functions (Jones and Harrison, 2001; Hoth et al., 2013; Aoki et al., 2014). Meta-analyses confirm that deficits in attention, long-term visual and verbal memory, visuospatial abilities, and working memory are more common in OSA patients than in healthy sleepers (Bucks et al., 2013). The presence of co-morbidities with OSA, such as stroke or age-related cognitive deficit, increases the severity of measured cognitive impairment, while prevalence of psychological disorders, like depression and anxiety, is also increased in OSA patients (Schröder and O'Hara, 2005; Kumar et al., 2009; Djonlagic et al., 2014; Aaronson et al., 2015; Acker et al., 2017).

At least two primary nocturnal abnormalities occur during OSA that may contribute to cognitive impairments: sleep fragmentation and intermittent hypoxia. While sleep fragmentation is considered the primary driver of daytime sleepiness in OSA, the majority of cognitive impairments appear to be due to intermittent hypoxia (Jackson et al., 2011). Dementia in elderly women with OSA and mild cognitive impairment in the apnea positive pressure long-term efficacy study (APPLES) has been correlated with severity of hypoxia as well (Quan et al., 2011; Yaffe et al., 2011). Deficits in attention and verbal episodic memory are also more pronounced in hypoxic patients as compared to non-hypoxic patients (Findley et al., 1988). Animal models have also demonstrated that impaired learning and memory is more closely associated with intermittent hypoxia than sleep fragmentation (Gozal et al., 2001; Polotsky et al., 2006). However, studies inducing sleep fragmentation in healthy adults in the absence of hypoxemia have resulted in daytime sleepiness and reduced sustained attention as well (Martin et al., 1996, 1999). Cognitive impairments in elderly patients with SDB have also been shown to associate with the severity of SDB, but

not the level of hypoxemia (Cohen-Zion et al., 2004). A recent study limiting the obstruction and intermittent hypoxemia of OSA to periods of REM sleep only demonstrated a reduction in spatial navigational memory, indicating the importance of REM sleep to memory in OSA patients, but could not separate the effects of intermittent hypoxemia or fragmentation during REM (Varga et al., 2014). Therefore, sleep fragmentation and intermittent hypoxemia are important contributors to cognitive impairment observed in OSA, but more work remains to be completed in order to determine the mechanisms by which OSA affects cognition.

1.4 Treatment of Sleep Apnea

Since the earliest identification of patients with Pickwickian syndrome, treatments to minimize patient symptoms have been sought. The first identified is still recommended today: weight loss. Enforced weight loss, by extreme calorie limitation, was the preferred treatment method to reduce or eliminate the effects of OSA in the mid 1900s (Burwell et al., 1956; Drachman and Gumnit, 1962). Ethical advances in medicine have led to physicians to no longer enforce weight loss as a method of OSA treatment but rather to only encourage dietary weight loss programs as a consideration. Aerobic exercise training is a popular treatment recommendation, although the mechanisms by which exercise induces OSA improvement is unclear; aerobic exercise training has been shown to reduce the severity of OSA, even in patients that exhibit little to no change in weight (Kline et al., 2011; Iftikhar et al., 2014). Advancements in bariatric surgery allow modern OSA patients that may be suffering from additional co-morbidities to quickly reduce excess weight; OSA patients that undergo bariatric surgery have reduced severity of OSA, a loss of OSA symptoms, and

reduced presence of diabetes at 2 year follow-ups, indicating outsized health benefits of weight loss in this population (Fritscher et al., 2007; Grunstein et al., 2007). Other surgical interventions are sometimes considered for younger patients with physical limitations of the upper airway (Tan et al., 2013; Garg et al., 2017). Tonsillectomy and uvulopalatopharyngoplasty remove excess tissue from the upper airway while maxillary-mandibular advancement moves the jaw bones further forward to enlarge the posterior pharynx, but these surgical interventions have wide variations in level of success (Khan et al., 2009; Caples et al., 2010). A tracheostomy is another option for surgical resolution or reduction of OSA symptoms, but both patients and physicians alike are reluctant to choose this option due to significant limitations on activity and unsightly appearance (Camacho et al., 2014).

While weight loss and surgical interventions have shown significant improvement in OSA patients, two devices are much more commonly prescribed as treatments and accepted by patients. The first is a mandibular advancement device (MAD), a hard-plastic device worn in the mouth during sleep that moves the jaw forward to widen the posterior pharyngeal airway. Meta analyses suggest that MADs are effective non-invasive treatment options for patients who suffer from mild OSA, with reported reduction of AHI by greater than 50% (Ramar et al., 2015). However, MADs are not as effective for patients with more severe OSA. They are particularly recommended for patients that do not tolerate the second, but most common, medical device for OSA treatment: positive airway pressure (PAP) machines.

First described in 1981, PAP provides pneumatic opening and patency of the upper airway (Sullivan et al., 1981). Available in continuous (CPAP), bi-level (BPAP), or

autotitrating (APAP) modes, PAP is the most effective treatment in reducing OSA severity and is the recommended treatment for moderate to severe OSA (Gay et al., 2006; Kushida et al., 2006; Epstein et al., 2009; McDaid et al., 2009; Iftikhar et al., 2017). CPAP treatment alleviates daytime sleepiness and fatigue by allowing for the restoration of REM and slow wave sleep (McDaid et al., 2009; Brillante et al., 2012; Gabryelska et al., 2017). Patients report improved quality of life after beginning routine CPAP therapy, with patients suffering from moderate to severe OSA reporting the greatest improvements (Batool-Anwar et al., 2016). CPAP use is associated with reduced mortality in middle and elderly aged males, after adjusting for comorbidities, and increased survival of the very elderly OSA patients (>80 years old) (López-Padilla et al., 2016; Jennum et al., 2017).

Cardiovascular and metabolic consequences of OSA are mildly reduced by the use of CPAP as well. Meta-analyses of adverse cardiovascular effects in OSA patients with CPAP treatment identify a reduction in blood pressure and, amongst those who use the device for greater than 4 hours a night, fewer major adverse cardiac events (McDaid et al., 2009; Moro et al., 2009; Bratton et al., 2015; Abuzaid et al., 2017). Data on CPAP treatment and metabolic disorders has been more mixed; small studies on OSA patients with diabetes mellitus have shown reductions in glycemic variability with CPAP treatment, while recent meta-analysis revealed that CPAP therapy provided no benefit to glucose metabolism in similar populations of patients. Both studies suggest that high variability in responses to therapy could be due to level of compliance with the CPAP device (Nakata et al., 2017; Zhu et al., 2017).

CPAP treatment has also been shown to mildly improve neurological outcomes in patients with OSA. Saunamaki and colleagues demonstrated that CPAP reversed trends in

reaction time, cognitive flexibility, and planning in OSA patients, but that working and language memory were unaffected (Saunamaki and Jehkonen, 2007). This data extends earlier reports that attentional deficits remained despite CPAP treatment, suggesting that OSA-induced cognitive deficits may be irreversible responses to intermittent hypoxemia (Kotterba et al., 1998). More recent studies have not been able to clarify which cognitive functions impaired in OSA are most sensitive to CPAP therapy. A few studies have demonstrated that CPAP treatment could improve verbal memory and executive function, but a recent meta-analysis from Kylstra and colleagues suggests that attention is the only cognitive function that is improved by CPAP (Antic et al., 2011; Kushida et al., 2012; Kylstra et al., 2013). A concurrent meta-analysis by Olaithe and Bucks surveyed five domains of executive function and found that all showed small to medium improvements with CPAP treatment (Olaithe and Bucks, 2013). One potential explanation for these varied responses to CPAP may be the populations of patients that were included in the studies, with some retaining more plasticity in brain regions responsible for memory and executive function. Cooke and colleagues interrogated cognitive ability in Alzheimer's patients suffering from OSA and show CPAP slowed cognitive deterioration (Cooke et al., 2009). Two additional and more recent studies focused on elderly OSA patients also show improved episodic memory, short term memory, and executive function, suggesting that populations most at risk for cognitive decline may show greater responsiveness to CPAP therapy (Crawford-Achour et al., 2015; Dalmases et al., 2015). An additional explanation for the wide range of reported responses to CPAP therapy is difficulty with compliance (Weaver and Grunstein, 2008). Despite advances in mask technology, humidity control, size and sound of CPAP machines, adherence has not greatly improved (Park et al., 2011; Lettieri et al., 2017). To

exclude the effect of insufficient treatment, several studies on the response to CPAP therapy stratified their results based on numbers of hours per night adherence. Dose responses were observed in sleepiness, verbal memory, and executive function, but a sub-population of OSA patients with high CPAP compliance continued to show no signs of cognitive improvement, suggesting that some OSA-induced deficits may be permanent (Weaver and Grunstein, 2008; Antic et al., 2011; Sforza and Roche, 2012).

1.5 Brain Regions Affected by Sleep Apnea

The types of cognitive impairments observed in OSA patients indicate the involvement of several brain regions, mostly in the cerebral cortex. The frontal cortex is implicated in multiple aspects of executive function, and has been shown to be susceptible to both sleep fragmentation and intermittent hypoxia (Morrell and Twigg, 2006; Funahashi and Andreau, 2013; Yuan and Raz, 2014). Using positron emission tomography (PET) scans, Thomas and colleagues found reduced glucose uptake in the prefrontal cortex, as well as in the thalamus and parietal cortex, in healthy patients subjected to sleep deprivation (Thomas et al., 2000). Extending these studies to OSA patients revealed qualitatively similar data on dorsolateral prefrontal cortex glucose uptake, with no change following CPAP treatment (Thomas et al., 2005). Santarneckchi and colleagues used functional magnetic resonance imaging (fMRI) in OSA patients and healthy age-gender-BMI-matched controls to generate cerebro-cerebellar regional homogeneity (ReHo) values used to identify pathology-related alterations in local coherence of low-frequency signals (Santarneckchi et al., 2013). The ReHo values in OSA patients were significantly reduced in regions within the prefrontal cortex, parietal cortex and cerebellum. Using T-1 weighted

MRI, Canessa and colleagues demonstrated that OSA patients have a reduction in grey matter volume within the frontal cortex, parietal cortex, and the hippocampus that correlated with reduced attention and executive function (Canessa et al., 2011).

Interestingly, CPAP treatment was able to specifically increase grey matter volume within clusters in the frontal lobe and hippocampus. Macey and colleagues also reported extensive white matter abnormalities in OSA patients using MRI (Macey et al., 2008). Together, these different imaging modality studies demonstrate that OSA patients have detectable changes in brain structure and function within the prefrontal cortex, which may contribute to the negative effects on executive function observed in these patients.

1.5.1 Sleep Apnea Affects the Hippocampus

The prefrontal cortex is a critical, but not exclusive, region of the brain for the exhibition of executive function. In addition to the prefrontal cortex, executive function is associated with the parietal cortex, temporal cortex, and subcortical nuclei (Frackowiak and Fletcher, 1996; Castronovo et al., 2009). Thomas and colleagues demonstrated that a subset of brain regions known as the executive network were activated when OSA patients treated with CPAP underwent tests of working memory, but not activated in OSA patients without treatment. The hippocampus, and the cortical regions adjacent to it, have been shown to exhibit abnormalities in neuroimaging studies of OSA patients. Studies by Morrell and Thomas using voxel-based morphometry of MRI show a reduction in grey matter in the left hippocampus of OSA patients that was later confirmed by studies conducted by Canessa and Torelli (Morrell et al., 2003; Thomas et al., 2005; Canessa et al., 2011; Torelli et al., 2011). Castronovo demonstrated the importance of hippocampus activation by fMRI

during a working memory examination in OSA patients (Castronovo et al., 2009). Recently, Kim and colleagues used deformation-based morphometry to show atrophy in several brain regions, including the hippocampus. CPAP treatment and treatment duration were associated with an increase in volume in a few subregions of the hippocampus, but no other deep grey matter structures (Kim et al., 2016). The OSA patients interrogated in the study showed pre-testing deficits in working memory, indicating that one or more of the brain regions that displayed atrophy is critical for this cognitive behavior.

While there has historically been debate about the role the hippocampus plays in within the limbic system, it is generally agreed upon that the hippocampus is critically important to learning and the formation of long-term memory (Lagali et al., 2010). Since the mid 1900s, epilepsy patients, such as the famous HM, have had portions of their hippocampi removed in order to treat disabling seizures. A portion of these patients subsequently suffered from amnesias, demonstrating that a major function of the hippocampus and its nearby cortical regions is to support the generation of new declarative memories (Shorvon, 2009; Knierim, 2015). Amnesic patients are able to retain memories from events prior to hippocampal damage or removal, and retain the ability to form new semantic memories, suggesting that while the hippocampus is important for forming new declarative memories, other brain regions contribute to memory storage and recall. More recent studies have attempted to identify the mechanisms by which the hippocampus contributes to memory formation. Goldfarb and colleagues identified a role for the hippocampus in guiding attention, while Jacobs and colleagues showed the ability of the hippocampus to detect elapsed time (Jacobs et al., 2013; Goldfarb et al., 2016). Yee extended these studies to spatial exploration and memory (Yee et al., 2014). These

functions are necessary contributors to the processes of pattern separation, temporal pattern separation, and spatial recognition memory (Yassa and Stark, 2011; Kesner et al., 2015). Animal models have aided in the elucidation of the mechanisms by which the hippocampus contributes to learning and memory. Gilbert and colleagues induced lesions within different subregions of the hippocampus and found that the different subregions were independently important for success on spatial and temporal pattern separation tasks (Gilbert et al., 2001). Jacobs et al used drug infusions to temporarily inactivate various hippocampal regions and demonstrated that these sites were necessary for discriminating small temporal differences, important for temporal pattern separation (Jacobs et al., 2013). Site specific hippocampal lesions were also used to identify the regions necessary for the recognition of novel objects and displays of anxiety-like behavior on the elevated plus maze (Hunsaker et al., 2008; Rice et al., 2015). Kalman extended these data by demonstrating that reduced hippocampal volume, following sciatic nerve injury in rats, was associated with disrupted social interactions in a resident-intruder test (Kalman and Keay, 2014). Together, these data indicate that the hippocampus is an important contributor to attention, recognition, spatial memory, and mood disorders. A unifying theory about how the hippocampus uniquely contributes to learning and new memory formation is that the ongoing process of neurogenesis within the dentate gyrus region of the hippocampus creates new substrates for these active functions.

1.6 Development and Structure of the Hippocampus

The hippocampus is a seahorse shaped structure located deep within the temporal lobe of humans, and is cashew shaped in rodents, lying superficially below the neocortex

(Knierim, 2015). There are two main histological divisions: cornu ammonis (CA), also known as Ammon's horn, and the dentate gyrus. The CA consists of primarily pyramidal neurons that produce glutamate, that can be separated by genetics and morphology into CA1 and CA3 regions (Lagali et al., 2010). The primary layout of these regions is recognizable as early as prenatal weeks 15-19 of human development (Arnold and Trojanowski, 1996). Using a mix of rodent and primate models, we know that pyramidal cell precursors arise during the end of gestation from the neuroepithelium and migrate in an inside-out fashion to the amniotic plate following their maturation (Angevine, 1965; Bayer and Altman, 1974; Nowakowski and Rakic, 1979; Altman and Bayer, 1990a). CA3 pyramidal neurons extend axons to synapse with the CA1 pyramidal neurons, referred to as Schaffer collaterals, and dendrites that receive input from the axons of dentate gyrus granule neurons, known as mossy fibers (Amaral and Dent, 1981). The granule neurons make up the primary cell layer of the dentate gyrus and are largely formed postnatally in rodents and primates (Bayer and Altman, 1974). In humans, this process includes a 4-6 month postnatal development period (Seress, 1992). Granule cell precursors migrate from the secondary dentate matrix near the neuroepithelium in two waves; the first occurs prenatally following the subpial route while the second occurs early after birth, migrating radially to the inner portion of the granular cell layer and the subgranular zone (Altman and Bayer, 1990b). The granular cell layer is split into a suprapyramidal and an infrapyramidal blade, with the space between them known as the hilus. The subgranular zone is a 2-3 cell thick layer between the granular cell layer and the hilus (Harry and D'Hellencourt, 2003).

The anatomical connectivity of the hippocampus is described as a trisynaptic loop upon cross-section along its long axis. The medial temporal lobe connects to the hippocampus through the entorhinal cortex to create the first synapse of perforant path projections onto dentate gyrus granule cells. The second synapse is where the mossy fibers of the DG project to the CA3 region. The third synapse connects CA3 to CA1 via Schaffer Collaterals, and CA1 projects back to the entorhinal cortex to complete the loop (Knierim, 2015). The unidirectionality of the trisynaptic loop was once believed to be entirely contained within a cross-sectional slice of the hippocampus; this hypothesis, known as the lamellar hypothesis, suggested that the hippocampus was simply a sum of layers of these cross-sections, each acting independently (Knierim, 2015). Anatomical tracing studies have since revealed vastly complex connection across the longitudinal axis, indicating that the cross-sections are not completely independent units within the hippocampus (Laurberg, 1979; Blessing et al., 2016). Interestingly, the entorhinal cortex sends projects directly to CA3 and CA1, which provide feedback signaling to the DG, contradicting the notion that the hippocampus is an exclusively unidirectional loop (Naber et al., 2001). Also, recent studies have shown that CA2 region has independent functions separate from a CA1-CA3 transition that are not usually considered part of the classic loop (Jacobs et al., 2013). Direct inputs from additional cortical regions, the perirhinal cortex and postrhinal cortex, along with major subcortical inputs from the raphe nucleus, locus coeruleus, medial septum, and amygdala demonstrate complex hippocampal circuitry connections that can contribute to various types of learning and memory (Laurberg, 1979; Knierim, 2015). However, the most popular theory regarding how learning and memory are manifested within the adult hippocampus is through neural stem cells that continually divide, differentiate into new

neurons, and functionally integrate into the dentate gyrus circuit (Gu et al., 2012; Park et al., 2015). Almost all neurons in the brain develop during gestation and mature shortly thereafter (Stiles and Jernigan, 2010). There are only a few sites of neurogenesis in the adult, and the high rate of cellular turnover in these regions contribute to the plasticity necessary for learning and new memory formation, discussed in detail in section 1.7 of this dissertation (Schmidt-Hieber et al., 2004; Ge et al., 2007).

1.7 Adult Neurogenesis in the Hippocampus

The hippocampus contains one of the few sites in the adult brain capable of adult neurogenesis. The subgranular zone of the dentate gyrus, the thin cell layer between the blades of the granular cell layer and the hilus, contains neural stem cells that continue to proliferate and differentiate following development. First identified in the 1960s, Altman and colleagues demonstrated the constitutive production of new hippocampal neurons using tritiated thymidine-labeling of dividing cells (Altman and Das, 1965). Kaplan and colleagues extended these data in the 1970s and 1980s using electron microscopy (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). The 1990s saw the identification of adult neurogenesis in a variety of model organisms, using the incorporation of bromodeoxyuridine (BrdU) and co-labeling with neuronal-specific markers such as NeuN or TuJ1 (Gratzner, 1982; Gage et al., 1995; Kuhn et al., 1996; Eriksson et al., 1998). More recently, Gage and colleagues have demonstrated that the newly born neurons can functionally integrate into the dentate circuitry, and that multipotent neural progenitor cells are responsible for adult neurogenesis in this niche (Gage, 2000; van Praag et al., 2002).

The neural progenitor cells reside in the subgranular zone (SGZ) and can be subdivided into active and quiescent populations (Suh et al., 2007; Lugert et al., 2010; Hodge and Hevner, 2011). Quiescent neural progenitor cells have a large soma in the SGZ and a long radial process that extends through the granule cell layer to branch out into many small processes in the molecular layer (Kempermann et al., 2004; Lugert et al., 2010). These cells divide at a slow rate and are therefore a small portion of total number of dividing cells. Active neural progenitor cells appear to have smaller somas and short processes that extend horizontally, remaining within the SGZ, and allow the cell to divide more frequently. Both subsets of cells express nestin, a marker of neural progenitors, and glial fibrillary acid protein (GFAP) a protein found in glia (Encinas et al., 2006; Suh et al., 2007; Lugert et al., 2010). Neural progenitor cells proliferate and can differentiate into glial cells or neurons, with several intermediate steps necessary before a cell can become an integrated and functional neuron. The intermediate progenitors, as they are known, remain mitotically active, but begin to express known neuronal markers, indicating their likely progression toward an ultimate neuronal fate (Hodge and Hevner, 2011). These cells also reside within the SGZ, often in clusters, and have few, if any, horizontal radial processes (Kempermann et al., 2004). There is more heterogeneity within the intermediate progenitor group, each cell exhibiting a unique mix of progenitor and neuronal markers. One subset of intermediate progenitors continues to express nestin, but does not express either GFAP or the microtubule-associated protein, doublecortin (DCX), found in developing neurons. A second subset is found to express nestin and DCX, while a third subset express DCX along with other neuronal markers, such as PSA-NCAM, without nestin co-expression. Since these subsets progressively increase their expression of neuronal

lineage markers while decreasing their expression of progenitor cell markers, it is likely that each subset of cells represents a step in a continual process of progenitor development, but the relationship among the three subsets remains to be elucidated completely (Hodge and Hevner, 2011). Newly-generated neurons in the dentate gyrus begin with their somas in the SGZ and extend dendritic processes through the granular cell layer toward the molecular layer. They continue to express DCX while these processes are extending, and transiently express calretinin, a calcium binding protein, for approximately 2 weeks in the rodent. During this time, the newborn neuron has either no synaptic input or exclusively GABAergic synaptic input (Overstreet-Wadiche and Westbrook, 2006). The newly-born neurons also express NeuN, a marker of post-mitotic neuronal nuclei, and soon after calretinin expression is reduced, the expression of an additional calcium-binding protein, calbindin, is ramped up. NeuN and calbindin can be found in adult and developmentally born granule neurons. During the transition period between calretinin and calbindin expression, the neuron grows larger in size, with greater number and circumference of dendritic branches, and the soma moves into the granular layer (Zhao et al., 2006). The neurons also begin to respond to glutamatergic stimuli and exhibit enhanced synaptic plasticity (Schmidt-Hieber et al., 2004). In rodents, it is estimated that it takes 4-7 weeks for new granule neurons to be generated and integrated into the circuit of the dentate gyrus (Kempermann et al., 2004; Ehninger and Kempermann, 2008).

1.7.1 Adult Neural Stem Cells

The self-renewing neural progenitor cells within the SGZ of the dentate are often referred to simply as neural stem cells, as it has been hypothesized that neural stem cells

are responsible for adult neurogenesis in germinal zones (Gage, 2000). Stem cells, in general, are undifferentiated cells that maintain multipotency, the ability to differentiate into several cell types. Pluripotent embryonic stem cells differentiate to yield several types of more specialized multipotent stem cells: hematopoietic stem cells yield blood cells only; mesenchymal stem cells yield osteoblasts, chondrocytes, and adipocytes; neural stem cells yield neurons and glial cells. The progenitor cells harvested from embryonic and adult hippocampi have been grown in culture to demonstrate their ability to self-renew and differentiate into multiple cell types (Ray et al., 1993; Palmer et al., 1995, 1997). *In vitro* analysis in both floating neurospheres and monolayer culture grown with supplemental growth factors revealed that at least some of the cells could expand substantially and undergo multiple passages while maintaining an undifferentiated state. When those growth factors were removed, the cells differentiated into a mixture of neurons, astrocytes, and oligodendrocytes, demonstrating multipotency. These data should be interpreted cautiously as the long-term exposure of growth factors could alter the behavior of many cell types, not just stabilize self-renewal in neural stem cells alone, and there is evidence that the self-renewal exhibited by these cells in culture is not unlimited (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005; Kippin et al., 2005; Furutachi et al., 2013; Ottone et al., 2014). Lineage tracing studies have shown neural progenitor cells *in vivo* to be capable of expansion and differentiation, although the full-extent of potential self-renewal was not determined (Dranovsky et al., 2011; Bonaguidi et al., 2012).

Independent of stem cell/progenitor cell naming controversy, adult neural progenitor cells do express similar genetic profiles to embryonic neural stem cells. Both express Hes5, a member of the basic-helix-loop-helix family of transcription factors that

maintains self-renewal and is often found alongside the more famous pluripotency marker, Sox2 (Lugert et al., 2010). Sox2 expression is also found in embryonic and adult neural progenitor cells, and is present in the majority of dividing cells in the SGZ (Ellis et al., 2004; Ferri et al., 2004; Suh et al., 2007; Lugert et al., 2010). Seldomly, Sox2 is expressed in cells that also express markers of neuronal fate commitment, such as DCX, indicating that Sox2 is downregulated as post-mitotic committed neural progenitors are produced (Ellis et al., 2004; Suh et al., 2007). Pax6, a paired domain and homeodomain-containing transcription factor, is expressed transiently in both embryonic generation of cortical glutamatergic neurons and in adult neural progenitor cells of the SGZ before differentiation into glutamatergic granule neurons. Expression is downregulated as embryonic or adult progenitor cells commit to the neuronal fate and begin to express DCX. Recently, it was demonstrated that astrocytes could be forced to act as neural progenitor cells in the striatum by viral induction of Sox2 expression alone, supporting the idea that Sox2 expression is integral to maintaining self-renewal and multipotency in all progenitor cells (Favaro et al., 2009; Niu et al., 2015).

The neural progenitor population is also regulated by several well-known extracellular signaling pathways. The signaling molecules that regulate embryonic nervous system development are conserved and continue to affect neural progenitor cell activity. The Wnt signaling pathway, a key regulator of proliferation and differentiation during embryonic development, has been shown to affect adult hippocampal neurogenesis; neural progenitors express several Wnts and corresponding receptors (Lie et al., 2005; Wexler et al., 2009). Sonic Hedgehog (Shh) signaling has also been demonstrated in developmental and adult neurogenesis; Shh rescues some non-proliferating neural progenitor cells and

loss of Smoothed, a downstream target of Shh, results in reduced neural progenitor cell proliferation (Han et al., 2008; Favaro et al., 2009). These signaling pathways can be activated by mature granular cells, astrocytes, oligodendrocytes, and/or microglia to regulate proliferation in neural progenitor cells (Suh et al., 2009; Lavado et al., 2010; Mu et al., 2010; Hodge et al., 2012). Interestingly, neural progenitor cells are also affected by hypoxia (Sims et al., 2009; Mazumdar et al., 2010; Varela-Nallar et al., 2014).

All cells must be able to sense and respond to fluctuations in oxygen concentration in order to balance energy generation and avoid unnecessary oxidative reactions, so it is no surprise that neural progenitor cells do so as well (Vieira et al., 2011). Embryogenesis requires physiologically low oxygen levels to regulate the proliferation and differentiation of stem cells (Ezashi et al., 2005). Mild sustained hypoxia has been shown to induce proliferation in neural progenitor cells in culture (Studer et al., 2000; Santilli et al., 2010). In contrast, severe hypoxia has been shown to arrest progenitor cell proliferation, leading to quiescence or apoptosis (Ezashi et al., 2005; Pistollato et al., 2007; Santilli et al., 2010). The oxygen concentration in the subgranular zone has been estimated to range from 2.5-3%, indicating that a neurogenic niche is also a hypoxic one. Despite high vascularization within the hippocampus, Mazumdar and colleagues, and later followed by Chatzi et al, have shown that neural progenitor cells reside within relatively hypoxic regions within the SGZ (Panchision, 2009; Mazumdar et al., 2010; Santilli et al., 2010; Chatzi et al., 2015). A high ratio of nuclei to blood vessels within the DG suggests that oxygen consumption rate could explain the levels of hypoxia experienced by neural progenitor cells (Mazumdar et al., 2010). Interestingly, Sun and colleagues demonstrated that SGZ cells committed to neuronal fate, by expression of neuronal markers, are in direct contact with blood vessels,

rather than undifferentiated neural progenitors (Sun et al., 2015). The presence of oxidative byproducts in committed neural progenitor cells within normoxic areas adjacent to hypoxic regions suggests that the transition of progenitors from a hypoxic niche to normal tissue oxygen levels contributes to both differentiation and cell death (Chatzi et al., 2015).

1.7.2 Adult-born granule neurons

Of the approximately 4,000 new cells born each day within the dentate gyrus of the mouse, only about 30% develop into mature granule neurons (Dayer et al., 2003; Kempermann et al., 2006; Snyder et al., 2009; Sierra et al., 2010). In humans, it is estimated that as many as 700 new neurons are born each day in the DG, with the vast majority of granule neurons undergoing exchange during adulthood, as compared to only 10% adult hippocampal turnover in rodents (Spalding et al., 2013). The high rate of cellular turnover in the region has led many scientists to hypothesize that adult neurogenesis is a necessary contributor to hippocampal-based learning and memory. While still under debate, analyses at the cellular, circuitry, and behavioral levels generate evidence in support of the critical contribution of newly-born neurons to hippocampal function (Ming and Song, 2011; Gu et al., 2012; Park et al., 2015).

At the cellular level, newborn neurons retain expression of calretinin, which may be related to different calcium handling in newborn neurons, such as the measurement of T-type calcium currents, which is not observed in mature granular cells. The resting membrane potential of these cells is notable greater than in mature neurons as well (Ambrogini et al., 2004; Schmidt-Hieber et al., 2004). At the circuitry level, newborn

neurons have been shown to have GABAergic responses that dissipate upon maturation (Overstreet Wadiche et al., 2005). Once new born neurons have formed glutamatergic synaptic connections, the cells exhibit increased synaptic plasticity (Overstreet-Wadiche et al., 2006; Ge et al., 2007). The threshold to induce long-term potentiation (LTP) within immature granule neurons is lower than that in mature neurons, and is only measurable from the newborn neurons that are not yet inhibited by local interneurons (Wang et al., 2000; Schmidt-Hieber et al., 2004; Saxe et al., 2006; Garthe et al., 2009). Temprana and colleagues recently demonstrated that newborn neurons poorly activate the interneurons responsible for feedback inhibition of granular cells, providing one mechanism for increased plasticity (Temprana et al., 2015). Interestingly, ablating newborn neurons through radiation results in reduced fEPSP slope, reduced population spike amplitude, and suppressed LTP, further demonstrating the importance of newborn neurons to synaptic plasticity in the dentate gyrus (Snyder et al., 2001; Park et al., 2015).

At the behavioral level, many studies have demonstrated a positive correlation between the degree of neurogenesis with performance in specific hippocampal-based behavioral tasks. Shors and colleagues used the antimetabolic agent MAM to block hippocampal neurogenesis and reported disrupted trace eye-blink and trace fear conditioning, but no change to contextual fear conditioning or spatial memory (Shors et al., 2001). Since then, several studies using irradiation have demonstrated hippocampal neurogenesis is important for contextual fear conditioning (Winocur et al., 2006; Warner-Schmidt et al., 2008; Hernández-Rabaza et al., 2009). Deficits to spatial memory following blockage of neurogenesis have also been shown by several groups; irradiation experiments completed by Madsen and colleagues revealed reduced place recognition but normal object

recognition, while Snyder et al. demonstrated deficits in a water maze (Madsen et al., 2003; Snyder et al., 2005). Qualitatively similar spatial memory deficits were generated using cytostatic agents and viral vectors to selectively ablate hippocampal neurons (Jessberger et al., 2009; Goodman et al., 2010). More recent studies have demonstrated deficits in spatial pattern separation, memory retrieval, and spatial memory discrimination following X-irradiation of young hippocampal neurons (Gu et al., 2012; Park et al., 2015). Conversely, increasing neurogenesis has been shown to be sufficient to improve pattern separation (Sahay et al., 2011). The use of several genetic methods to silence adult neurogenesis have extended these data in spatial learning and memory to show deficits in spatial relational memory acquisition, context encoding, context discrimination, and performance in the Barnes and water mazes (Dupret et al., 2008; Imayoshi et al., 2008; Zhang et al., 2008; Danielson et al., 2016).

Immature granule neurons have been shown to be preferentially incorporated into circuits supporting spatial memory and independent roles for young and older adult-born neurons have been identified in pattern separation and completion, thus providing a mechanism by which spatial memory is driven by adult neurogenesis (Kee et al., 2007; Nakashiba et al., 2012). Together, these studies reveal that newly-born neurons aid in the plasticity necessary for learning and memory.

1.8 Conditions that alter Adult Neurogenesis

Adult neurogenesis is sensitive to a variety of environmental stimuli. Physiological, pathological, and pharmacological challenges have been shown to regulate the production of newborn neurons in the adult hippocampus (van Praag et al., 2005; Zhao et al., 2008;

Lazarov et al., 2010). Neural progenitor cell proliferation in the SGZ is highest during youth, and decreases with age (Kuhn et al., 1996; Nacher et al., 2003; Rao et al., 2006). Trophic factors such as fibroblast growth factor 2 (FGF2), insulin-like growth factor-1 (IGF-1), and glial-derived neurotrophic factor (GDNF) can induce proliferation in the SGZ (Aberg et al., 2000; Chen et al., 2005b; Dictus et al., 2007). Conversely, transforming growth factor beta (TGF- β) and interleukin-1 β (IL-1 β) negatively impact adult neurogenesis (Wachs et al., 2006; Koo and Duman, 2008; Bauer, 2009). Unsurprisingly, trophic factors that promote proliferation are decreased in hippocampi of older animals, while anti-proliferative factors are increased (Miyazaki et al., 2003; Shetty et al., 2005). Physical enrichment, in the form of wheel running, can attenuate age-related decline of neurogenesis in rodents (van Praag et al., 1999a, 1999b; Steiner et al., 2004). Mental enrichment, in the form of enhanced rodent housing environments, has also been shown to increase neurogenesis (Kempermann et al., 1997; Steiner et al., 2004). A variety of pharmaceutical treatments have been shown to impact neurogenesis. Chemotherapy that targets quickly dividing cells expectedly reduces adult neurogenesis, although voluntary wheel running can attenuate neuronal loss and even some memory deficits associated with the cancer treatment (Winocur et al., 2014). Antipsychotic medications have been shown to promote neuronal differentiation using human neural stem cells in culture and antidepressants are reported to do so within the hippocampus itself (O'Leary and Cryan, 2014; Asada et al., 2016; Åmellem et al., 2017). NMDA and GABA receptor antagonists are associated with increased neurogenesis, while their respective agonists reduce neurogenesis (Nacher et al., 2003; Maekawa et al., 2009; Nakamichi et al., 2009; Giachino et al., 2014). Hormones such as erythropoietin and adrenal corticoids are also reported to increase neurogenesis, showing that proliferation and

neuronal differentiation in the adult SGZ are sensitive to many environmental stimuli (Arabpoor et al., 2012; Lehmann et al., 2013).

In congruence with physiological and pharmacological stimuli, several pathological conditions can alter adult neurogenesis. Addiction and commonly abused drugs, such as cocaine, methamphetamine, and alcohol, have been shown to negatively impact the development of new neurons in the dentate gyrus (Venkatesan et al., 2007; Yuan et al., 2011; Geil et al., 2014; Castilla-Ortega et al., 2017). High-fat diet has been shown to impair neurogenesis through a lipid peroxidation mechanism (Park et al., 2010). Two different animal models of diabetes display reduced hippocampal neurogenesis, suggesting that new neuron formation in diabetes patients may suffer synergistically along with the influence of poor diet (Jackson-Guilford et al., 2000; Beauquis et al., 2008; Guo et al., 2010). Neurological disorders, such as Alzheimer's disease and Parkinson's disease, are also associated with decreased neurogenesis, independent of the neuronal loss that defines each disorder (Mu and Gage, 2011; Marxreiter et al., 2013). Although many pathological conditions are known to reduce neurogenesis, there are several that are linked with an increase in newborn neurons in the dentate gyrus. Aberrant neuronal firing observed in epilepsy is associated with increased cell proliferation and differentiation; following disruption of neurogenesis using irradiation, seizures have been shown to restore neurogenesis and fear memory (Warner-Schmidt et al., 2008; Jessberger and Parent, 2015). Stroke also significantly stimulates neurogenesis in many brain regions, including those that do not normally undergo adult neurogenesis; however, the morphologies of the newly-generated granule neurons are abnormal and do not induce improvements in spatial memory (Lindvall and Kokaia, 2015; Woitke et al., 2017). In a similar fashion to stroke, traumatic brain injury can

induce increases in adult neurogenesis within the dentate gyrus (Bye et al., 2011; Zheng et al., 2013; Zhang et al., 2015c). However, studies that extend neurogenesis monitoring out past the length of time needed for a single cycle of neurogenesis reveal that traumatic brain injury ultimately results in fewer new-born neurons, suggesting that post-injury neurogenesis may undermine neurogenic potential and promote aberrant cell development (Bye et al., 2011; Neuberger et al., 2017). In conclusion, multiple pathological conditions, including those that alter tissue oxygenation, can affect the development of neurons in the adult hippocampus in multiple ways over extended periods of time.

1.9 Summary

Sleep is necessary for healthy neurological function and sleep apnea is a disorder that can result in non-restorative sleep. Since its first identification in the mid-1900s, obstructive sleep apnea has become a major health care concern, affecting up to a quarter of American adults, particularly those with a combination of significant risk factors such as obesity, male sex, and advanced age. The periodic cessation of breathing during OSA results in a multitude of pathological responses, ranging from mild snoring to the acceleration of cardiovascular and neurological disease. OSA impacts both the autonomic nervous system, as demonstrated by cardiorespiratory abnormalities, and the central nervous system, as shown by cognitive deficits. CPAP therapy alleviates many of these symptoms, but does not address the underlying cause of sleep apnea-induced neurological dysfunction. One potential mechanism that has been underexplored is how newly born hippocampal neurons, and the neural progenitor cells that they differentiate from, respond to sleep apnea. As one of only two sites capable of adult neurogenesis, the function of the hippocampus is not only dependent upon

the electrochemical signals of its primary neurons, but also the development and integration of new neurons into its circuitry. Despite data from sleep apnea patients and animal models indicating that the hippocampus may be particularly susceptible to damage from repeated periods of hypoxemia, there have been no extensive studies on the function of adult hippocampal neurogenesis during sleep apnea.

Chapter 2

Reasoning and Hypothesis

Obstructive sleep apnea is a highly-prevalent respiratory disorder characterized by periods of sleep during which no effective breaths are taken (Burwell et al., 1956). Defined by obstruction in the upper airway, these recurrent episodes of apnea result in repeated bouts of hypoxemia (Quan et al., 1997). Approximately a quarter of Americans are at risk for developing sleep apnea, with increased age and weight being the greatest risk factors (Peppard et al., 2013). One third of American adults are considered obese, and that number is anticipated to rise along with the number of adults that enter middle and old age, thereby likely increasing the prevalence of sleep apnea as well (Bhattacharjee et al., 2012). Patients with this disorder have a greater risk of developing cardiovascular complications, such as hypertension and stroke (Somers et al., 2008). With the current cost of diagnosing and treating sleep apnea in the US estimated to be \$12.4 billion per year, sleep apnea also represents a considerable economic burden for the American population (Watson, 2016).

Sleep apnea negatively impacts the function of the central nervous system independent of cardiovascular complications (Dewan et al., 2015). Patients with untreated sleep apnea complain of fatigue, demonstrate reduced cognitive function, and can have alterations in the activity and structure of various brain regions (Bloom et al., 2009; Canessa et al., 2011). One region consistently reported to be affected in sleep apnea patients is the hippocampus (Torelli et al., 2011).

The hippocampus is a critical regulator of learning and memory and one of only two structures in the mammalian brain capable of producing new neurons throughout an individual's lifetime (Lieberwirth et al., 2016). Newly-generated immature neurons are effective substrates for Hebbian plasticity and display unique properties when compared to their relatively older counter parts (Ge et al., 2007; Gu et al., 2012; Park et al., 2015). The generation of immature neurons in the hippocampus is disrupted in several disease conditions where cognitive decline is observed, indicating that adult neurogenesis plays an integral role in hippocampal-based learning and memory (Lazarov et al., 2010; Mu and Gage, 2011; Marxreiter et al., 2013). Therefore, injury to the neurogenic niche of the hippocampus may be a significant contributor to the cognitive impairment observed in sleep apnea patients. While oxygenation state plays a critical role in the regulation of adult neurogenesis (Mazumdar et al., 2010; Chatzi et al., 2015), little is known about how intermittent hypoxia, a principal consequence of sleep apnea, mechanistically impacts hippocampal function.

The overall goal of this thesis project is to gain first insights into how sleep disordered breathing impairs the cellular based mechanisms of learning and memory in the hippocampus. To this end, I designed experiments to test the function of the hippocampus under sleep apnea using the examination of hippocampal-dependent behavior and synaptic plasticity within the hippocampal circuit. I also queried the sensitivity of adult neurogenesis to sleep apnea through immunohistological studies of both neural progenitor cells and newly-born neurons. These experiments test the unifying hypothesis that intermittent hypoxia causes dysfunction in multiple stages of adult neuronal development and function, thus contributing to hippocampal injury.

Chapter 3

Materials and Methods

3.1 Chronic Intermittent Hypoxia

In order to effectively mimic the clinical presentations of OSA, I utilized chronic intermittent hypoxia (CIH) in mice. CIH has been used as a model of OSA since at least as far back as 1992 (Fletcher et al., 1992), but there has been little consistency in the pattern, timing, severity, and duration of hypoxia exposures (Davis and O'Donnell, 2013). The specific CIH protocol utilized in these studies, described in detail below, results in oxygen desaturation of 60-80% in a pattern similar to mild/moderate forms of OSA (AHI 10/hr) (Dumitrascu et al., 2013) and has been published routinely since 2003 (Peng and Prabhakar, 2003; Kumar et al., 2006; Kumar and Prabhakar, 2008; Peng et al., 2011; Garcia et al., 2016).

Rodent models of CIH recapitulate a wide range of cardio-respiratory and neurophysiological pathologies observed in the clinical presentation of OSA, although historically most research has focused on the cardiac effects. Fletcher and colleagues were the first to show IH could induce hypertension in rodents (Fletcher et al., 1992). In a severe and frequent protocol of IH, they also demonstrated that continued IH led to left ventricular hypertrophy, and implicated the carotid body as the mechanism by which IH

induced cardiopathologies. In 2001, Fagan followed up on this study with a focus on the pulmonary circulation and presented qualitatively similar data; IH induced pulmonary hypertension and right ventricular hypertrophy, further implicating IH as a contributor to heart failure in OSA patients (Fagan, 2001). Using echocardiography, the standard method of determining heart function, Chen and colleagues indeed showed left ventricular dilation, decreased ejection fraction, and decreased cardiac output following CIH (Chen et al., 2005a). More recently, an increase in cardiomyocyte diameter and the presence of interstitial fibrosis in left ventricular myocardium were also identified following CIH, further supporting an increased risk of heart failure in these models (Hayashi et al., 2008). Further contributing to cardiac dysfunction, Savransky et al first described the induction of atherosclerosis by CIH in 2007, when paired with a high-cholesterol diet (Savransky et al., 2007). Animals eating high-cholesterol diets without IH and CIH-exposed animals with a normal diet did not develop aortic atherosclerotic plaques. Follow-up studies have since shown that CIH accelerates the progression of existing atherosclerosis and can induce the formation of lesions in the absence of other risk factors (Song et al., 2015). Taking a more detailed focus, Tahawi and colleagues studied the effects of CIH on the vasculature in the cremaster muscle of male rats and demonstrated that CIH reduced the vasoconstriction responses to both acetylcholine and L-NAME, a stereospecific inhibitor of nitric oxide synthase (NOS) (Tahawi et al., 2001). This vascular dysfunction was confirmed in follow-up studies by Phillips and colleagues focused on the resistance arteries of the gracilis muscle. Resting tone, myogenic activity, and vasoconstriction in response to norepinephrine (NE) were all reduced in CIH-exposed rats (Phillips et al., 2006). The reduced vasoconstriction activity is of particular interest following the demonstration by Kumar and colleagues that

CIH induced increased systemic catecholamine levels (Kumar et al., 2006). The adrenal medullae from CIH-exposed rats secreted increased NE and epinephrine, and were unable to respond to additional stimulation by nicotine, similar to findings in OSA patients (Elmasry et al., 2002). As catecholamines have been implicated in the progression of atherosclerotic plaques (Born, 1991), these studies may provide a mechanism that explains the prevalence of atherosclerosis in CIH patients in addition to recapitulating the symptoms. Closely mimicking the clinical presentation of OSA, the rodent models of CIH demonstrate vascular dysfunction, atherosclerosis, hypertension, ventricular remodeling, and heart failure all observed in the clinical setting.

Models of CIH in rodents also recapitulate the neurological symptoms observed in OSA patients. First, mice and rats can serve as model species because they naturally exhibit central apneas, periods without breaths and no intercostal muscle activity, as determined by plethysmography and intercostal muscle electromyography (EMG) (Christon et al., 1996; Nakamura et al., 2003). Next, oxygen desaturation leads to repeated arousal from sleep in rodents (Polotsky et al., 2006). Gozal and colleagues demonstrated that CIH results in a temporary disruption in both rapid eye movement (REM) and non-REM (NREM) sleep, as categorized in the clinic (Gozal et al., 2001; Zinchuk et al., 2017). These disruptions were diminished by the second day of exposure to CIH, and did not lead to chronic sleep deprivation. Qualitatively similar data by Polotsky and colleagues showed that a model of sleep fragmentation with the same frequency and duration as CIH did not disrupt REM and NREM sleep, supporting the use of CIH to model sleep disturbances observed in patients (Polotsky et al., 2006). In fact, Veasey and colleagues describe a “sleepy” phenotype in rodent models of CIH, with the loss of wake active neurons resulting in hypersomnolence

and increased susceptibility to short term sleep loss (Veasey et al., 2004; Zhu et al., 2007). Sleep disturbances such as these result in alterations to cognitive executive function, just as OSA is reported to do (Naegele et al., 1995; Durmer et al., 2005). For example, Decker et al demonstrated deficits in working memory in CIH-exposed neonate rats tested in the radial arm maze test while Gozal and colleagues have repeatedly shown CIH-induced deficits in swim mazes (Gozal et al., 2001, 2010; Decker et al., 2003; Row et al., 2003; Kheirandish et al., 2005). By demonstrating sleep arousal by hypoxia, alterations to REM/NREM sleep, increased somnolence, reduced executive function, and reduced spatial memory, rodent models of CIH recapitulate much of the neuropathology observed in OSA patients.

Our model of CIH consists of alternating gas exposures into standard mouse caging, and was approved the Institute of Animal Care and Use Committee (IACUC) of Seattle Children's Research Institute. In the ALAAC-approved facility, mice were housed in Thoren caging with *ad libitum* access to both food and water, and were maintained on a 12-hour light/12-hour dark cycle, in accordance with National Institutes of Health (NIH) guidelines. Computer-controlled solenoid valves allowed for 80 rounds of intermittent hypoxia during a single light-cycle, and normoxia during the dark-cycle. Intermittent hypoxia consisted of 60 seconds of 100% Nitrogen gas, during which nadir Oxygen reached $5 \pm 1\%$ for approximately 5 seconds, followed by an immediate flush of room air (21% Oxygen gas) for 300 seconds. Normoxia was achieved no later than 60 seconds post-room air flush. Chronic IH was defined as 30 days of alternating gas exposures, and will be referred to as IH₃₀.

3.2 Animals

Adult male and female mice, aged P29-P35, were used for all experiments. All littermates were exposed to CIH or normoxia simultaneously, as the gas exposures were limited to a single home cage. Two strains of mice were used: the standard C57BL/6J (The Jackson Laboratory cat: 000664), and a tamoxifen-inducible reporter strain maintained on a C57BL/6J background: Nestin-Cre/Ai27D reporter. See Section 3.3 of this dissertation for more information regarding the reporter mouse strain. Littermates not carrying the reporter system were also exposed to CIH, but not used for birth-dating experiments. All strains were pair mated in house to provide sufficient numbers of experimental animals.

3.3 Genetic birth-labeling of neural progenitor cells

In order to effectively identify neural progenitors in the dentate gyrus, mice expressing Cre recombinase protein bound to human estrogen receptor ligand-binding domain with a G400V/M543A/L544A triple mutation (cre/ERT2) under the Nestin promoter were bred to mice carrying the Ai27D reporter (Sun et al., 2014). Ai27D mice harbor the transgene for channelrhodopsin bound to tD-tomato fluorescent protein downstream from a *loxP*-flanked STOP cassette and under the Rosa26 promoter (Madisen et al., 2012). Both strains of mice are commercially available at The Jackson Laboratory (Nestin-Cre/ERT2 cat: 016261; Ai27D cat: 012567). Mice were genotyped to ensure at least hemizygous expression of both Cre and Ai27D, prior to inclusion in the study (see table 3.1 for primers and table 3.2 for PCR and electrophoresis protocols). Littermates not carrying both genes were also exposed to CIH, but not used for birth-dating experiments. Mice carrying least one copy of both genes were given two intraperitoneal injections of tamoxifen at a dose of 180mg/kg, dissolved in corn oil. Each injection was given 18 hours

apart over the two consecutive days immediately prior to the start of CIH or normoxia exposure.

Sequence Name	Number of Bases	Sequence (5'-3')	Tm (50mM NaCl) C	Expected product size (bp)
Cre32	20	GAA GCA TGT TTA GCT GGC CC	56.74	300
Cre52	23	GTC CAA TTT ACT GAC CGT ACA CC	55.53	
Ai14; F	23	TCG TGA TCT GCA ACT CCA GTC TT	58.15	homo: 196 hetero: 196 and 297
Ai14; R	22	TGG GCT ATG AAC TAA TGA CCC C	56.49	

Table 3.1: Primer Sequences. All primer sequences used to genotype Nestin-CreER^{T2};Ai27 mice are listed above. DNA samples from experimental mice were amplified in PCR reactions using the sequences above, according to the protocols shown in Table 3.2, and separated via 1.5% agarose gel electrophoresis. Bands observed at 196 bp for Ai and 300 bp for Cre were considered positive for presence of each gene. Mice carrying at least one allele of both genes of interest (homo- or hemi-zygous) were included in the study.

Reaction conditions							
Cre				Ai14			
Step	Temp (C)	Time (sec)	Cycles	Step	Temp (C)	Time (sec)	Cycles
Denaturing	95	120	1	Denaturing	95	180	1
Denaturing	95	30	30	Denaturing	95	30	30
Annealing	62	30		Annealing	62	40	
Extension	72	30		Extension	72	40	
Final Extension	72	600	1	Final Extension	72	600	1

Table 3.2: PCR protocol for amplification of Cre and Ai DNA. DNA was isolated from tail samples of mice to be genotyped, and amplified according to the conditions listed above. Cre protocol yields a 300bp product while Ai14 protocol yields a 196bp product when separated via 1.5% agarose gel electrophoresis (130V for 30 mins).

3.4 Behavioral Assessments

3.4.1 Open field Assessment and Novel Object Recognition

Following 26 days of CIH or normoxia control, individual animals were transferred to commercially-sold open field assessment arenas (Noldus or Plexon) to test exploratory activity and anxiety during the light cycle (Prut and Belzung, 2003; Stanford, 2007). A

camera, mounted above the arena, was used to record the mice during a 10-15 minute period of non-restricted arena exploration, aka habituation (Noldus or Plexon). On the subsequent day (27D CIH/control), animals were returned to the arena for novel object familiarization: two similar objects (geometric solids, Learning Resources) were added to the arena and animals were allowed to explore for a 15 minute period. On the subsequent day (29D CIH/Control), animals were returned to the arena for a 15 minute testing period along with one of the objects used during training and an object new to the animal. Subsequent exploration was videotaped in order to test object recognition memory (Antunes and Biala, 2012). Automated animal tracking was used to measure the distance travelled and to create a heat map of where each animal spent the greatest time while in the arena (Plexon CineLab and Noldus EthoVision).

3.4.2 Barnes Maze

Following 26 days of CIH or normoxia control, individual animals were transferred to a custom-made Barnes Maze apparatus to test spatial reference memory (Sharma et al., 2010). The apparatus consisted of an elevated circular surface (92 cm dia., 30cm from floor), with 20 evenly-spaced exit holes (5 cm dia.) and an escape box. To prevent subjects from making spatial associations between distal room cues and the location in the maze (Harrison et al., 2006), the maze was placed in a walled arena (wall height 60 cm). Illumination of the maze was consistent for each experiment. A testing protocol modified from Rosenfeld and Ferguson (2014) was used (Rosenfeld and Ferguson, 2014). In brief, subjects underwent three training sessions followed by one test session. Each session was 6 min in duration and separated by a 24 hr period. Subjects were placed in a transport box

that was positioned in the maze center prior to the start of each session. Each session began immediately upon removal of the box and subjects were allowed to explore the arena. During the training sessions, one hole was open (i.e., the exit zone) leading to the escape box. The apparatus was cleaned with ethanol and the exit zone moved to a different hole in between each individual subject. Mice unable to locate the exit zone during a training session were guided towards the zone and allowed to enter the escape box where they stayed for one minute. During the test session (30D CIH/Control), the escape box was removed and all holes were closed. Tracking was performed using CineLAB software (Plexon Inc., Dallas TX). The total distance traveled, latency to the first entry into exit zone, and the percentage of visits spent exploring defined zones were assessed.

3.5 Antioxidant treatment

To determine the effect of oxidative stress in the CIH model, a subset of mice exposed to CIH were treated simultaneously with daily intraperitoneal injections of the cell-permeable superoxide anion scavenger, 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)-21H,23H-porphyrin manganese(III) pentachloride, (MnTMPyP; 15mg/kg). This group is referred to as IH_{MnTMPyP}. MnTMPyP is one member of a class of metalloporphyrin catalytic antioxidants that have been reported to have superoxide, hydrogen peroxide, and peroxynitrate scavenging activity (Liang et al., 2009a; Batinic-Haberle et al., 2012). Our laboratory and others have used this antioxidant to show reduced oxidative stress in hypoxic or ischemic nervous tissue injuries (Sharma and Gupta, 2007; Celic et al., 2014; Garcia et al., 2016). A cohort of mice exposed to normoxia were also injected with MnTMPyP daily to serve as controls, and are referred to as Control_{MnTMPyP}.

3.6 Electrophysiology

3.6.1 Tissue Preparation

To test action potential generation, a cohort of animals was anesthetized with isoflurane and decapitated, according to IACUC-approved protocols. Coronal sections (450 μ m) collected through the hippocampus by Leica LT1000s vibratome were immediately incubated in 30mM glucose artificial cerebrospinal fluid (aCSF). Sections were continuously bubbled with 95% O₂/ 5% CO₂ at room temperature for a minimum of one hour prior to any recordings to allow for recovery from the dissection. The external solution (aCSF) contained the following (in mM): 118 NaCl, 30 Glucose, 25 NaHCO₃, 3.0 KCl, 1.5 CaCl₂, 1.0 NaH₂PO₄, and 1.0 MgCl₂ (305-315 mOsm).

3.6.2 Paired-pulse Facilitation Protocol

To examine paired pulse facilitation, the fEPSP was evoked every 20 sec with interpulse intervals of ranging from 20ms to 500ms. The paired pulse ratio (PPR) at each interpulse interval was calculated according to the equation below:

$$PPR = \frac{m_2}{m_1}$$

where m_2 is the m_i evoked by the second stimulus pulse and m_1 is the m_i evoked by the first stimulus pulse.

3.6.3 LTP Induction Protocol

A bipolar stimulating electrode (0.1- 0.4ms; 200- 400mA) was placed into the perforant path and a recording electrode was placed into the dendritic roots of the dentate

gyrus. Recorded signals were amplified 10,000 times, filtered (low pass, 1.5 kHz; high pass, 250Hz), rectified, and integrated using an electronic filter. To block potential inhibitory transmission, the specific GABA receptor blocker, picrotoxin (25uM), was added to the bath at least 10 min prior to any recording (Olsen, 2006). Individual synaptic responses were elicited at 20-second intervals. After establishing baseline recordings (approximately 10 min), LTP was induced by high-frequency stimulation (HFS): train of 50 pulses at 100 Hz repeated four times at 30-second intervals. Excitatory post-synaptic potential (EPSP) recordings were acquired in pCLAMP software (Molecular Devices, Sunnyvale, CA) and continued for up to an hour following HFS. Recordings were analyzed posthoc in Clampfit software (version 10.2).

3.7 Tissue Processing and Histology

Mice were terminally anesthetized according to IACUC- approved protocols, transcardially perfused with saline and followed by 50mL of 4% paraformaldehyde at a constant pressure and volume. Brains were dissected and post-fixed in 4% paraformaldehyde overnight. The tissue was then cryoprotected in 30% sucrose for a minimum of 2 days until equilibrated and frozen in blocks of optimum cutting temperature (OCT) medium by super-cooled ethanol. Blocks containing a single hemisphere from each animal were sectioned at a thickness of 40 μ m on a Leica cryostat, and stored in a cryoprotectant solution of primarily glycerol at -20° C. Immunohistochemistry was performed on floating sections using fluorescent dye-conjugated secondary antibodies, as previously described (Hodge et al., 2008, 2012). All protocols included an overnight, approximately 18 hour, exposure to the primary antibodies used and a two-hour exposure

to fluorescently-conjugated secondary antibodies. Some antigens required additional retrieval using 0.1% citrate buffer solution prior to exposure to primary antibodies (see table 3.3 for details). Every 12th section was sampled, ensuring each animal in the study had at least three usable sections through the septal region of the dentate gyrus that contained both the suprapyramidal and infrapyramidal blades. A standard staining protocol can be found in Figure 3.1.

Antibody	Host	Supplier	Catalog #	Dilution	Antigen retrieval
anti-Doublecortin	Goat	Santa Cruz	sc8066	1:250	no
anti-GFAP	Mouse	Millipore	MAB360	1:20,000	no
anti-ki67	Rabbit	Vector	VP-RM04	1:500	no
anti-RFP	Rabbit	Rockland	600-401-379	1:500	no
anti-Sox2	Goat	Santa Cruz	sc17320	1:250	1 boil in citrate buffer
anti-synaptopodin	Rabbit	Synaptic Systems	102002	1:500	no
anti-Tbr2	Rat	eBioscience	14-4875-82	1:500	2 boils in citrate buffer

Table 3.3: Primary Antibody Information. All primary antibodies used in the studies are listed above. Tissue was incubated overnight for approximately 16 hours. See Figure 3.1 for full protocol.

Antibody	Host	Supplier	Catalog #	Dilution
anti-Goat-IgG-488	Donkey	Invitrogen	A11055	1:400
anti-Goat-IgG-647	Donkey	Invitrogen	A21447	1:400
anti-Mouse-IgG-647	Donkey	Invitrogen	A315171	1:400
anti-Rabbit-IgG-488	Donkey	Invitrogen	A21206	1:400
anti-Rabbit-IgG-568	Donkey	Invitrogen	A10042	1:400
anti-Mouse-IgG-488	Donkey	Invitrogen	A21202	1:400

Table 3.4: Secondary Antibody Information. All secondary antibodies used in the studies are listed above. Tissue was incubated for 2 hours in 1:400 dilution of secondary. See Figure 3.1 for full protocol.

Project: _____ Date: _____

Protocol for 2-day Floating Immunofluorescent Staining

Updated: 03/10/15 CMP

DAY 1

1. _____ PBS (5mins)
2. _____ PBS (5mins)
3. _____ PBS (5mins)
4. _____ Antigen Retrieval? (30mins total)
 - a. _____ Heat in Sodium Citrate Buffer (8 mins)
 - b. _____ Allow to cool to room temperature (10-15 mins)
 - c. _____ PBS (5mins)
 - d. _____ PBS (5mins)
5. _____ Blocking solution (60 mins)
6. _____ Primary Ab (prepare in Block) Antibody: _____ Host: _____
Antibody: _____ Host: _____

** Shake overnight **

DAY 2

7. _____ PBS (20mins)
8. _____ PBS (20mins)
9. _____ PBS (20mins)
10. _____ Secondary Ab (2 hours: prepare in Block) Antibody: _____ Host: _____
Antibody: _____ Host: _____
11. _____ PBS (20mins)
12. _____ PBS (20mins)
13. _____ PBS (20mins)
14. _____ DAPI (2 min)
15. _____ PBS (5mins) (Store in PBS until mounting) _____
16. _____ Mount tissue on slides from a solution of ½ PBS ½ H₂O
17. _____ Dry slides at room temperature (10-15 mins)
18. _____ Coverslip using Fluormount G mounting media
19. _____ Cure overnight

Solutions:

PBS (Phosphate buffered saline pH 7.4)
10x PBS
Make 1L: 100mL 10x into 900mL MilliQ water

Sodium Citrate (0.01M or 10mM, pH 6.0)
Can re-use
Make 500mL:

Blocking Solution (PBS 0.1% Triton X-100, 10% serum)
Host: _____
1ml Triton X-100 per 1L PBS
1mL serum per 10mls PBS 0.1% Triton X-100

Antibody Information:

Host, Antigen:

Source, catalog, lot:

Dilution:

Figure 3.1: Immunofluorescent staining protocol: Step-by-step instructions for the implementation of immunofluorescent labeling of antigens listed in Table 3.3. Antigen retrieval, step 4, was skipped unless staining for Sox2 (x1) or Tbr2 (x2).

3.8 Image collection and Quantitation

Single plane images of all sections containing usable dentate gyrus (see Section 3.7) were captured at low magnification (10x, 0.8 N.A. air objective) on a Zeiss LSM 710

confocal microscope using Zen software. Low magnification images of DAPI (Sigma-Aldrich) and synaptopodin, a synaptic vesicle protein enriched in the axons of dentate granular neurons, were used to quantify the volumes of hippocampal subregions. The granule cell layer (GCL) was determined by the area stained by DAPI, the hilus was defined as the area between the suprapyramidal and infrapyramidal blades of the dentate gyrus labeled by DAPI, and the mossy fiber track was defined by the entire area stained with synaptopodin. All regions of interest were measured using Zen software (Zeiss). Volumes (V) were estimated using Cavalieri's principle, $V = \sum A_i \cdot d$; taking the sum of aforementioned areas (A) multiplied by the interval (i) and the distance (d) between sections sampled (Rosen and Harry, 1990; Prakash et al., 1994; van Praag et al., 1999b; Chatzi et al., 2015).

Z-stack images were obtained for all other immunohistochemical stains within the entire section of usable dentate gyrus using a 40X, 1.3 N.A. oil objective on the same Zeiss LSM 710 confocal microscope with Zen software, and were quantified using Image J software. Multiple images were required to capture the complete dentate gyrus within each usable section. The section's entire region of interest, across multiple images, was counted. Cells intersecting the top-plane of each image were excluded. Cells per dentate were estimated again according to Cavalieri's principle: raw counts for all imaged sections were multiplied by the interval (i) and the distance (d) between sections sampled (Rosen and Harry, 1990; Prakash et al., 1994; van Praag et al., 1999b; Chatzi et al., 2015). For counts of DCX⁺ cells, immature neurons were defined as having a cell body located in the SGZ and a radial process extending through the GCL. Cell counts were limited to the SGZ neurogenic niche for proliferating cells, neural stem cells, and intermediate neural progenitor cells. The

SGZ region of interest was defined as a 2-3 cell thick layer between the GCL and hilus, as previously described (Miller et al., 2013).

To explicitly label a discrete cohort of the progenitor cells, pulse labeling experiments were performed using mice carrying both Nestin-cre/ERT2 and Ai27D genes, as described in Section 3.3 above. Immunostaining for red fluorescent protein (RFP) was used to identify cells positive for the td-tomato reporter molecule. Triple immunostaining for RFP along with glial fibrillary acid protein (GFAP) and doublecortin (DCX) were used to divide the birth-labeled cells into four categories: (1) RFP⁺ immature granule neurons (2) GFAP⁺ and RFP⁺ cells; (3) DCX⁺ and RFP⁺ progenitor cells; (4) RFP⁺ only non-neuronal cells. RFP⁺ immature granule neurons were defined by RFP positive staining and morphology with radial processes emanating from the soma and extending into the GCL. Some, but not all, RFP⁺ immature granule cells also expressed DCX. RFP⁺ only non-neurons exhibited neither clear radial processes nor co-labeled with GFAP or DCX.

Sholl Analysis was conducted on fully visible neurons selected from each experimental group and imaged at high magnification (100x, 1.46 NA oil objective) on a Zeiss LSM confocal microscope (Binley et al., 2014). Images were compressed into a maximum intensity projection in ImageJ (NIH) (Schindelin et al., 2012; Schneider et al., 2012). Using the Simple Neurite Tracer ImageJ plugin, dendritic paths of individual neurons were traced and analyzed with the Sholl Analysis plugin (available in FIJI) (Schindelin et al., 2012). Concentric circles were drawn around the cell body in 10 μ m increments and the number of neurite intersections with each circle was calculated. Intersections were plotted as a linear function of radius to serve as a measure for neurite

complexity. Analysis was limited to birth-labeled neurons expressing RFP and at least one dendrite of 120 μ m length from the soma.

Statistics were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Comparisons between two groups were conducted using unpaired t-tests with Welch's correction, to account for unequal variances between groups. For comparisons involving more than two groups, a one-way ANOVA was performed followed by a *post hoc* Dunnett's test. Unless otherwise stated, data are presented as mean \pm S.E.M. Significance was defined as *P < 0.05, **P < 0.01, and ***P < 0.001. Analyses that were not statistically significant were defined as "ns".

Chapter 4

Results and Discussion

4.1 Thirty days of IH reduces exploration, object recognition, and spatial memory

Untreated OSA patients demonstrate a variety of deficits in cognitive executive functions, including reduced attentional capacity, short-term memory, working memory, and long-term memory (Naegele et al., 1995; Canessa et al., 2011). To test whether exposure to chronic intermittent hypoxia results in similar cognitive difficulties observed in OSA patients, we evaluated the behavior of animals exposed to either 30 days of intermittent hypoxia (IH₃₀) or normoxia-exposed controls in a hippocampal dependent behavioral test: open field assessment (Prut and Belzung, 2003). The two groups travelled a similar distance in the open field (Figure 4.1A top left), indicating no differences in locomotor activity. When the pattern of locomotion was quantified, IH₃₀-exposed animals entered the center of the field significantly fewer times than control mice ($p=0.0457$) (Figure 4.1A bottom left). Representative heat maps for each group are presented in the right-hand side of Figure 4.1A. Normal rodent exploratory behavior would consist of initial preference for the edges of the arena, followed later by entries into the center of the arena, as they habituate to the space. These data indicate CIH exposure reduces habituation and may indicate an alteration in anxiety-like behavior (Seibenhener and Wooten, 2015) Recent studies have revealed a sub-

region of the dentate gyrus is altered in anxiety-based behaviors, so we sought to determine whether other hippocampal-dependent tasks were also affected by CIH (Kheirbek et al., 2013; Weeden et al., 2015).

Several behavioral assessments in mice are available to interrogate the health and function of the hippocampus. To test exploratory behavior and object recognition memory, we utilized the novel object recognition test (Broadbent et al., 2010). Following habituation and familiarization over a period of two days, mice were exposed to a known object and a novel object and had their interactions recorded. Normal mouse exploratory behavior includes a preference for exploring a new object over one that the mouse has been habituated to. When tested, the groups spent similar periods of time exploring the objects, indicating no changes in exploratory drive or anxiety. Both groups displayed a slight preference for the novel object and did not exhibit significant differences in the percentage of novel object interactions (Figure 4.1B), indicating that CIH does not affect novel object recognition. Mice with cortical lesions consistently fail to show a preference for the new object (Antunes and Biala, 2012). Wide variation in hippocampal injury models have shown mixed results on novel object recognition, with both no changes and mild deficits reported. A recent review suggests that the hippocampus is indeed an important region for recognition memory, but that its relative contribution can be muted by compensation from other brain regions if the hippocampus suffers large, permanent damage (Cohen and Stackman, 2015). Our novel object recognition data is consistent with studies of hippocampal injury where the injury is mild and temporary, not unlike the data from OSA patients treated by CPAP therapy described in Chapter 1 (Yuet et al., 2010; Olaithe and Bucks, 2013). Further supporting the role of that the hippocampus plays in recognition memory, Kesner and colleagues performed

complex object-spatial feature configuration recognition tasks on rats with hippocampal lesions. Their data suggests that, specifically, the dentate gyrus region of the hippocampus retains object-placement and complex object-place feature information, which are both necessary for spatial memory (Kesner et al., 2015).

In order to more accurately test the effects CIH has on spatial recognition and memory, we conducted a Barnes maze assessment on CIH or normoxia exposed mice. Over three days of training, the control group exhibited decreased latency to the exit zone, while the IH₃₀ group failed to demonstrate a significant decrease in latency to the exit zone over the training period, indicating CIH may affect spatial learning (Figure 4.1C, left and middle). During the testing period, the total distance traveled was similar between groups (Control: 27.6±1.7 m, n=10 vs. IH₃₀: 22.7±2.1 m, n=9) indicating that CIH does not cause locomotor deficits. Additionally, the IH₃₀ group required more than twice the amount of time to reach the escape hole than normoxia-exposed control mice and exhibited greater variability (Figure 4.1C right), indicating failure to demonstrate spatial memory (Sharma et al., 2010). Although animals in both control and CIH groups displayed a preference for the exit zone, the percentage of exit zone entries was significantly greater when compared to other defined zones in the maze for the control group (Figure 4.1D left), indicating precision in spatial memory. Yet, in the IH₃₀ group, the percentage of exit zone entries was similar to an adjacent zone (Figure 4.1D right), likely an expression of reduced precision in spatial memory. While other brain regions are also important for exploratory behavior, recognition memory, and spatial memory, these data, taken together, suggest that CIH induces mild deficits to hippocampal-dependent behavior.

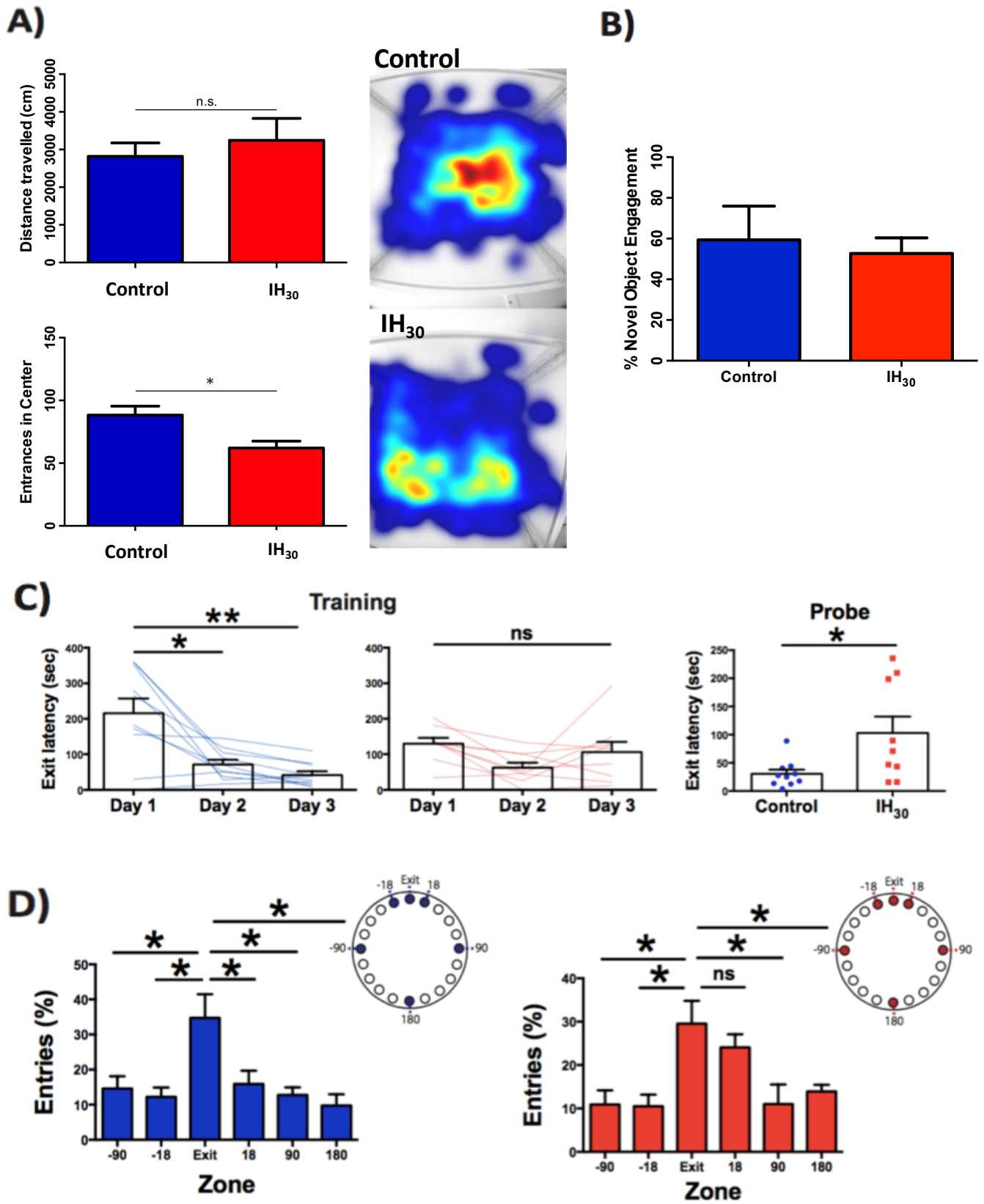


Figure 4.1: IH₃₀ animals exhibit deficits in select learning and memory tasks.

(A) During non-guided exploration, IH₃₀ mice (n=3) entered the center of an open field fewer times than control (n=3) (bottom left, $p=0.0457$). Mice travelled similar distances during non-guided exploration (top left, $p=0.5725$). Representative heat maps from each group shows that Control mice spent more time (red) in the center of the open field than IH₃₀. **(B)** Control (n=3) and IH₃₀ (n=3) mice show a similar preference for a novel object in the novel object recognition task ($p=0.7385$). **(C)** After three successive training days, control animals (blue line, n=10) exhibited decreased latency to the exit zone of the Barnes Maze (Left-hand side, $p=0.0027$). Dunnett's multiple comparison *post hoc* analysis revealed a significant decrease in latency between Training Day 1 and Training Day 2 as well as Training Day 1 and Training Day 3. Comparatively, IH₃₀ animals (middle, red line, n=9) did not exhibit decreased latency during the training days of the Barnes Maze test ($p=0.1193$). During the probe trial of the Barnes Maze test (right-hand side), the latency to the exit zone was smaller in control (n=10) compared to IH₃₀ (n=9) ($p=0.0392$). **(D)** One-way ANOVA revealed the percentage of exit zone entries ($100 \times (\text{number of entries} / \text{sum of entries})$) was greater than the other five sampled zones in the control group (n=10, $p=0.0025$, all comparisons to exit zone significant) (left-hand side). In contrast to control, the IH₃₀ group (right hand side, n=9) exhibited less precision for locating the exit zone as the percentage of exit zone entries was similar to an adjacent zone ($p=0.0033$, adjacent zone not significant).

4.2 Thirty days of IH suppresses long term potentiation (LTP) in the dentate gyrus

Synaptic plasticity has been considered the neurochemical foundation of learning and memory since at least the mid-twentieth century, when Lømo and Andersen first demonstrated long-term potentiation (LTP) in the rabbit hippocampus (Lømo, 1966). In the time following, many groups have demonstrated that that hippocampal specific LTP is necessary, but not sufficient, for demonstrating long term memory (Martin et al., 2000; Kim and Diamond, 2002; Izquierdo et al., 2008; Neves et al., 2008). The strongest pieces of supporting data are from studies where LTP is blocked and deficits in learning were observed (Morris, 1989). The behavioral data we acquired showing CIH altered exploratory behavior and spatial memory is qualitatively similar to data presented by Gozal and colleagues, and implies that global hippocampal circuitry may be susceptible to CIH-induced deficits (Payne et al., 2004).

In order to test the function of the hippocampal circuit, we performed electrophysiological recordings on *ex vivo* slices of brain tissue containing the dentate gyri from animals exposed to 30 days of IH or normoxia. We stimulated the lateral perforant pathway to induce synaptic plasticity and recorded field potentials from the dendrites of the granular neurons of the dentate gyrus. We examined both paired-pulse facilitation and LTP of field excitatory postsynaptic potentials (fEPSPs). The profile of paired-pulse facilitation of the fEPSP was not different between groups at any interpulse interval examined (Figure 4.2A), suggesting that presynaptic facilitation of medial perforant path was unaffected by CIH. However, following tetanic stimulation, the fEPSP was potentiated in the control group (n=9; blue line), yet suppressed following IH₃₀ (n=6; red line) (Figure 4.2B left). Following a high-frequency stimulation (HFS) protocol to induce LTP at this synapse, we also observed

fEPSP potentiation in animals that underwent a 10-day exposure to IH (10D CIH). No differences in the potentiated slopes of fEPSPs normalized to baseline between slices prepared from Control or 10D CIH mice (Supplemental Figure 4.1). The percentage of slope change remained consistent over the course of an hour in both groups, consistent with previous reports on dentate gyrus LTP following short term CIH exposure (Wall et al., 2014). However, after IH₃₀, we observed an immediate and significant reduction in the potentiated fEPSP slope following HFS (Control: 185% of baseline vs. IH₃₀: 130% of baseline; Figure 4.2B). Moreover, the slope reduction continued over the remaining time course of HFS experiments, revealing that CIH suppressed LTP expression in the dentate gyrus (Figure 4.2B). Our data is qualitatively similar to experiments performed by Gozal and colleagues; they demonstrate that CIH negatively impacts synaptic plasticity in the CA1 subfield of the hippocampus, a region important for spatial memory consisting solely of neurons generated during development (Payne et al., 2004). Since this region also showed increased apoptosis under CIH, a deficit in LTP induction and maintenance implicates neuronal cell loss as a potential mechanism by which CIH negatively impacts hippocampal-dependent cognition.

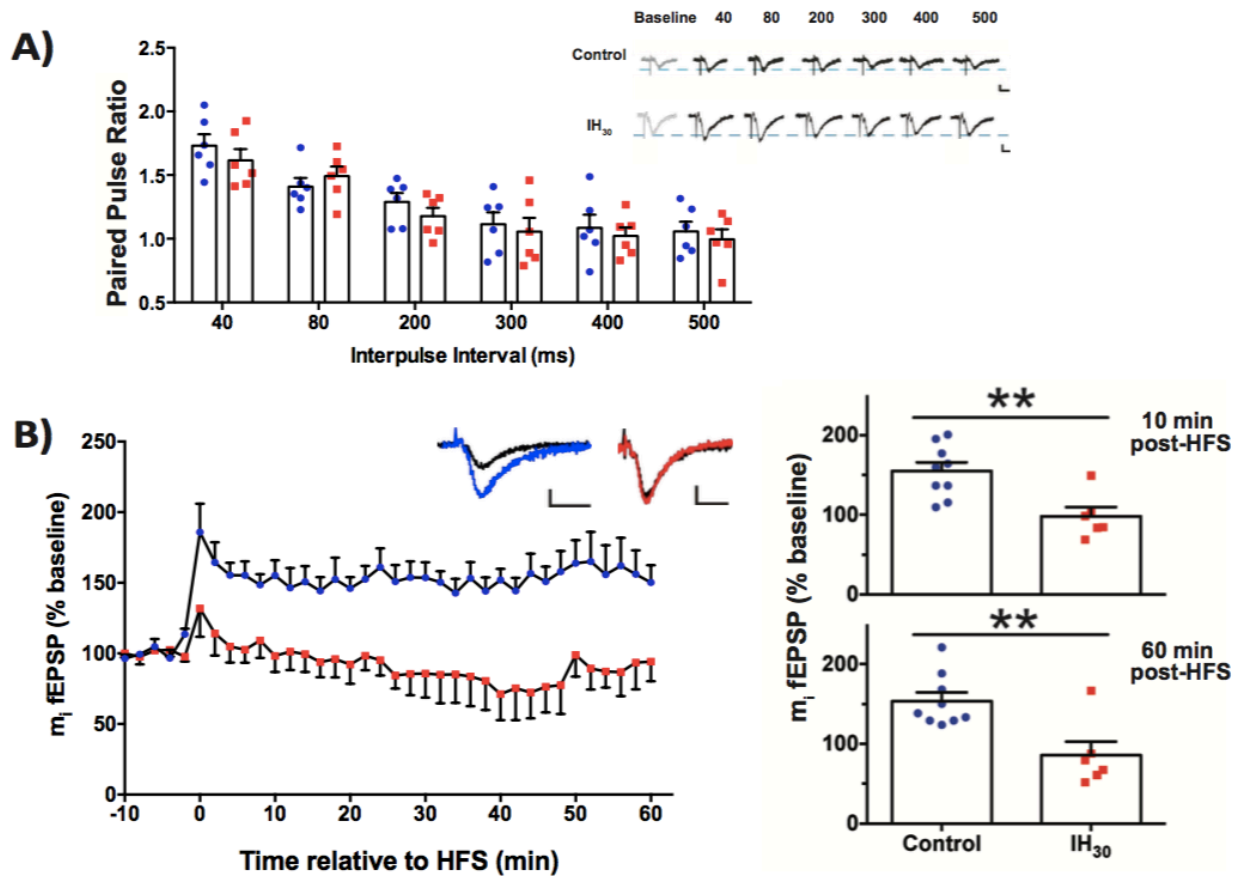


Figure 4.2 *IH₃₀ attenuates LTP and does not affect paired-pulse facilitation within the dentate gyrus.*

(A) Paired pulse facilitation of the fEPSP was similar between Control (blue circles, n=6) and IH₃₀ (red squares, n=6) at all six interpulse intervals tested ($p=0.7259$). Representative traces of evoked fEPSPs are shown right of the graph. Scale bars: 0.2mV x 10ms. **(B)** LTP of the fEPSP following high frequency stimulation (HFS) in control (blue circles, n=9) and IH₃₀ (red squares, n=6) showed significant difference between control and IH₃₀ 10 mins post-HFS ($p=0.0036$) and 60 mins post-HFS ($p=0.0086$). Representative traces of evoked fEPSPs are shown above the graph with baseline (black trace) and post-HFS induction indicated (color traces: Control= blue; IH₃₀= red). Scale bars: 0.2mV x 10ms.

4.3 CIH does not affect macroscopic hippocampal structure.

From Parkinson's disease, to dementia, and ageing, multiple clinical pathologies demonstrate that a loss of neurons is identifiable by a reduction in brain volume (Bigler and Tate, 2001; Peters, 2006; Lin et al., 2013). We sought to determine whether the deficits we observed in hippocampal-dependent cognitive assessments and synaptic plasticity were associated with a change in the gross structure of the hippocampus, including its neurogenic niche. Following immunohistochemical labeling of synaptopodin counterstained with DAPI, the areas of several subregions of the hippocampus were measured and volume estimated using the cavalieri method (Rosen and Harry, 1990; Prakash et al., 1994). No differences between CIH and normoxia exposed groups were found when comparing the estimated volume of DAPI staining within the granule cell layer (Table 4.1) or in the estimated volumes of synaptopodin staining in the hilus nor the mossy fiber tract (Table 4.1).

Region	Control	IH ₃₀	p-Value
Granule Cell Layer	0.171±0.022	0.189±0.008	0.488
Hilus	0.212±0.031	0.331±0.104	0.318
Mossy Fiber Tract	0.279±0.036	0.324±0.020	0.325

Table 4.4: Volume of Select Hippocampal Structures

No differences in macroscopic hippocampal structures were identified between mice exposed to Control (n=4) or IH₃₀ (n=6). All values are given as mean volume ± SEM (mm³).

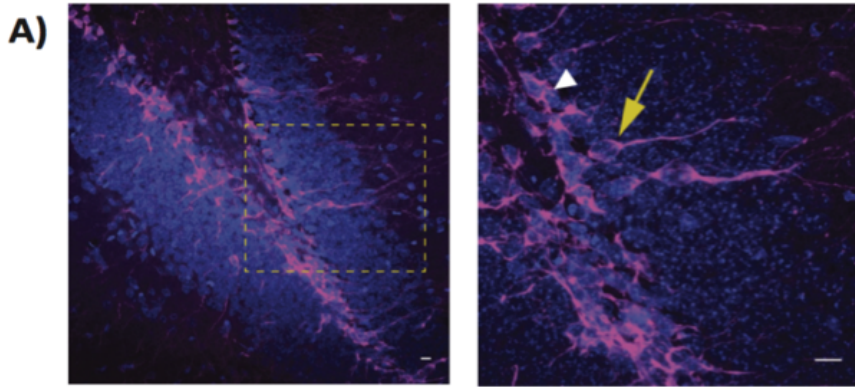
No change in the volume of these regions indicates that CIH does not induce a gross loss in the total number of granular neurons nor the number, length, or width of the axonal projections from those neurons. While some pathological conditions are associated with

reduced hippocampal volume in human patients, other neural pathologies with serious manifestations simply do not demonstrate significant brain volume loss. Depression and cognitive decline are both associated with decreased hippocampal volume (Frodl et al., 2006; Elcombe et al., 2015). Temporal lobe epilepsy patients have displayed a mix of both no change as well as reduction in hippocampal volume, but interestingly, all demonstrate a loss in NeuN positive neurons within the hippocampus (Peixoto-santos et al., 2015). Adrenalectomy in rats results in similar losses of hippocampal granular cells, that are demonstrated by a reduction in hippocampal granular volume (Sloviter et al., 1989). Selective loss of GABA-ergic cells in the hippocampus of the engrailed-2 null mouse, a model of autism, results in no change in hippocampal volume yet still recapitulates deficits in social interaction, locomotion, spatial learning, and memory (Sgadò et al., 2013). Our findings indicate that gross neuroanatomical volumes of the dentate gyrus and surrounding regions are unaffected by CIH, but do not discount the potential impact CIH may have on individual cells in the granule layer or adult neurogenesis.

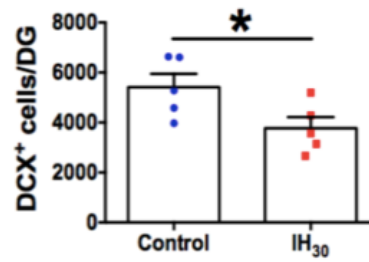
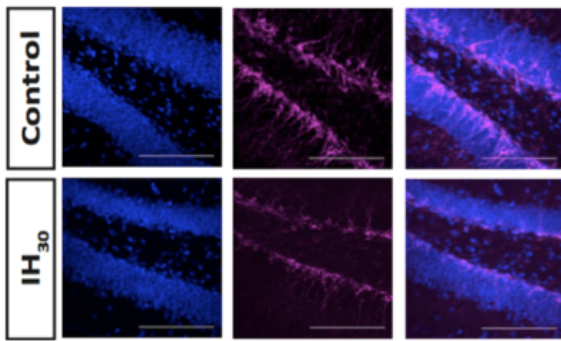
4.4 CIH decreases the number of adult-born neurons in the dentate gyrus.

Cognitive deficits and a failure to induce LTP implicates neuronal cell loss as a potential mechanism by which CIH negatively impacts hippocampal-dependent cognition. In fact, attenuated LTP is correlated with a reduction in neurogenesis in the dentate gyrus (Snyder et al., 2001). In order to determine whether CIH affects new adult-born neurons, the number of doublecortin-positive (DCX⁺) cells with projections entering into the granule cell layer were examined. DCX, a microtubule-associated protein, is expressed in neuronal precursor cells, both intermediate progenitor cells and immature granular neurons

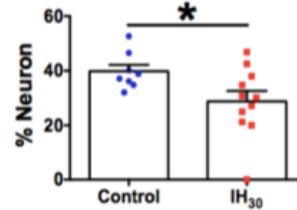
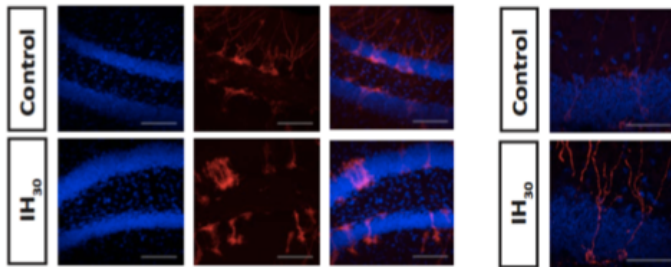
(Gleeson et al., 1999). By restricting positive cell counts to those cells with a dendritic projection (Figure 4.3A yellow arrow), we counted only the newly-generated neurons in the DG. CIH decreased the number of DCX⁺ cells (Figure 4.3B) by 30.4% when compared to the control group. This suggests that CIH suppressed the generation of adult-born granule neurons. In addition to counting DCX⁺ cells, we also performed a series of pulse labeling experiments using nestin-creERT2;Ai27D mice to determine the fate of birth-labeled progenitor cells in the SGZ (Supplemental Figure 4.2). After IH₃₀, the percentage of td-tomato positive (td-tomato⁺) neurons out of the total number of td-tomato⁺ cells in the dentate gyrus was reduced to 28.8±3.8%, down from 39.8±2.3% in control (Figure 4.3C) and represented an approximate 28% decrease in the proportion of birth-labeled adult-born neurons. To describe the morphology of new-born neurons developed under CIH, we performed a Sholl analysis, which revealed no differences in the number or complexity of dendritic branches between neurons developed under CIH or control conditions (Figure 4.3D). Together, our findings indicate that CIH affects the number of adult-born neurons, but not necessarily the dendritic trees of surviving neurons.



B) **DAPI/ DCX⁺/Merged**



C) **DAPI/RFP⁺/Merged**



D)

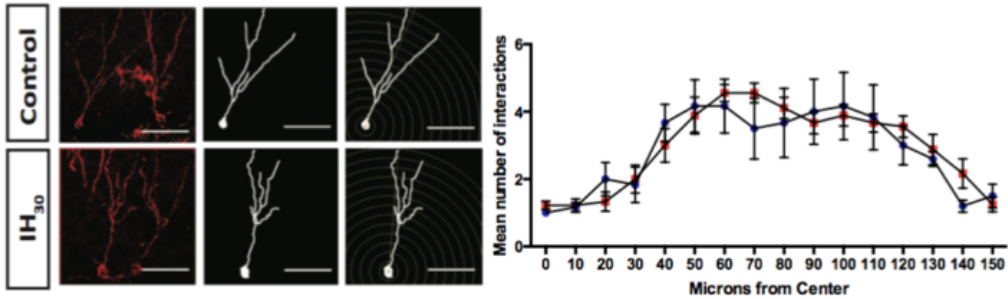


Figure 4.3 *IH₃₀ reduces the number of newborn neurons without affecting dendritic branching.*

(A) Representative image of DCX+ labeled cells at low and high magnification. The yellow arrow indicates a DCX+ immature neuron that was included in the analysis based on morphology. The white triangle points to an excluded DCX+ cell. Scale bars: 10 μ m **(B)** IH₃₀ (n=5) reduced the number of DCX+ cells (pink) with neuronal morphology as compared to Control (n=5) (p=0.046) Scale bars: 100 μ m **(C)** IH₃₀ (n=9) results in fewer RFP-labeled neurons (red) than in Control (n=9) (p=0.017) Scale bars: 100 μ m, left; 50 μ m, middle **(D)** Sholl analysis revealed no difference in dendritic branching between Control (n=7) and IH₃₀ (n=10). Concentric circles were separated at 10 μ m intervals around the cell body of each neuron. Scale bars: 50 μ m.

The newborn neuron population is an important contributor to synaptic plasticity and memory in the hippocampus (Snyder et al., 2001; Schmidt-Hieber et al., 2004; Ge et al., 2007; Park et al., 2015). Snyder and colleagues described two pharmacologically distinct forms of LTP in the rat dentate gyrus, an NMDA-sensitive higher LTP amplitude form and an NMDA-insensitive lower LTP amplitude form, consistent with a more sensitive developing and a less-responsive established population of neurons, respectively (Snyder et al., 2001). Schmidt-Heiber and colleagues expanded on this data in 2004 by separating the two neuronal populations by input resistance and presence of polysialic acid neural cell adhesion molecule (PSA-NCAM) immunohistochemical labeling. Young granular neurons differed substantially from mature granular neurons in both active and passive membrane properties that favored action potential generation with small stimuli (Schmidt-Hieber et al., 2004). Qualitatively similar data was presented by Ge and colleagues, using a retroviral neuronal birthdating strategy; young granular neurons were hyperexcitable, required a lower threshold to achieve LTP, and demonstrated an increased LTP amplitude (Ge et al., 2007). In a more recent study, the same group was able to selectively silence young adult born-neurons, which resulted in a reduced retrieval of hippocampal memory (Gu et al., 2012). Ablating newly-born cells in the dentate by either X- or gamma-irradiation, led to a reduction in fEPSP slope, reduced population spike amplitude, blocked LTP, and reduced memory retrieval (Snyder et al., 2001; Park et al., 2015). Several groups also demonstrate the importance of newly-born neurons to the integration and recall of recognition and spatial memory (Bruel-Jungerman et al., 2005; Tashiro et al., 2007). Bruel-Jungerman and colleagues show that rats in enriched environments have more BrdU-labeled neurons and increased recognition memory, and that both effects are blocked by the addition of an anti-

mitotic drug (Brüel-Jungferman et al., 2005). The Gage group expanded these data in mice and identified a critical period, approximately 2 weeks after neuronal birth, during which the establishment of spatial memory was the greatest (Tashiro et al., 2007). Similarly, Kee and colleagues utilized immunohistochemical birth labeling to demonstrate the preferential recruitment of adult-generated neurons into the spatial memory networks necessary to complete the Morris water maze (Kee et al., 2007). Our electrophysiological and behavioral data, failure to induce LTP in IH₃₀ mice (Figure 4.2B) and increased time to exit zone in the Barnes maze (Figure 4.1C, D), is consistent with a loss of contribution from a young granular neuron cohort. Indeed, we demonstrate fewer immature neurons following CIH-exposure and a reduced number of neurons that developed during IH₃₀ (Figure 4.3B,C), providing a potential mechanism by which CIH affects hippocampal memory.

Our observation that CIH negatively impacts the number of newly-born neurons initially appears to contradict previous studies of CIH and neurogenesis. Gozal and colleagues reported a biphasic effect of CIH on neurogenesis in the rat, based on a combination of BrdU and neurofilament double-labeling. They demonstrate a decrease at 7 days of CIH or less, and an increase at both 14 and 30 days of CIH, which correlated with an early deficit in Morris water maze testing that was not detectable by 14 days (Gozal et al., 2003). One important difference between their study and the present one is the methodology used to demonstrate proliferation in newly born neurons; the authors used daily injections of BrdU throughout the entirety of CIH exposure, thus labeling multiple cohorts of developing neurons, and compared that to singly-injected controls. Our study follows a single cohort of developing neurons to demonstrate that IH impacts adult neurogenesis through reducing the number of newly-born neurons that develop. In

addition, the type of intermittent hypoxia utilized was different from that in the current study; Gozal alternates 90 seconds of 10% oxygen with 90 seconds of normoxia, differing in both severity and patterning. This model may produce a less severe injury that the rat can compensate for, as supported by the lack of differences between CIH and normoxia controls in behavioral tests at later time points. In 2016, Pedroso and colleagues reported that a more similar CIH model increased neurogenesis in the rat, based on increased proliferation and neural stem cell markers in the dentate gyrus when compared to a different strain of rat with a salt-diet induced model of hypertension. However, fewer neurons were actually identified by NeuN and Map2 labeling at the same time, consistent with the IH₃₀ data presented in the present study. Pedroso's study describes CIH as a model of hypertension that is distinct from the commonly used salt-diet sensitive rat model, but does not completely eliminate hypertension as a confounding factor. Spontaneously hypertensive rats have been shown to have reduced grey matter and reduced neurofilament staining in the dentate gyrus, consistent with Pedroso's findings on DG neurons (Sabbatini et al., 2002). Also demonstrated in both spontaneous and CIH-induced hypertension models is an increase in GFAP labeling within the dentate, which could be associated with either neural stem cells or astrocytes (Tomassoni et al., 2004). Using markers of proliferation, such as BrdU and Ki67, alone to demonstrate increased neurogenesis, when these markers could be present in other non-neuronal-fated cells, can be slightly misdirecting. The double-immunolabeling paradigms of a proliferation marker paired with a neuronal marker used by both Gozal and Pedroso provide a better method for determining the birth of new neurons than proliferation alone, but also targets the aggregate generation of neurons from multiple cycles of neurogenesis occurring over the

period of CIH. Our genetic birth-labeling paradigm allowed us to follow the proliferation and differentiation of a discrete population of neural stem cells over the course of CIH exposure and to determine that fewer of those NSCs developed into neurons. However, the end points that we utilized cannot discriminate between IH-induced apoptosis of neurons, IH-induced neural stem cell contraction, or an IH-induced shift towards gliogenesis.

4.5 CIH increases mitotic division, stem cell population, and neural progenitor population in the subgranular zone (SGZ) of the dentate gyrus.

The source of newly-born adult neurons in the dentate gyrus are endogenous neural stem cells (NSCs), which are highly responsive to environmental stimuli (Lazarov et al., 2010). Insufficient NSC expansion is one potential explanation for how CIH reduces the number of immature granular neurons in the dentate gyrus. To determine whether CIH reduced mitotic division within the neurogenic niche, we stained for Ki67, a cell cycle protein present during interphase (Scholzen and Gerdes, 2000). Limiting the quantitation to the SGZ, we show that, paradoxically, Ki67 labeling in the SGZ region of DGs of IH₃₀ mice is nearly double that of normoxia-exposed controls ($p=0.047$) (Supplemental Figure 4.3). As discussed above, multiple groups have previously reported an increase in hippocampal proliferation following CIH (Gozal et al., 2003; Zhu et al., 2005, 2010; Pedroso et al., 2016). Our data is consistent with these studies, as well as reports from other types of brain injury within the dentate gyrus (Jin et al., 2006; Rola et al., 2006). This finding suggests that CIH causes an increase in proliferation within the neurogenic niche; however, the identity of these proliferating cells remains unclear. Glial cells proliferate in response to inflammation and, since CIH has been shown to induce mild inflammation, could be responsible for the

increased proliferation we observed (Monje et al., 2003; Sapin et al., 2015). In order to determine whether the neural stem cell population is expanding under CIH, we stained for the pluripotency marker, Sox2 (Ellis et al., 2004). Sex determining region Y-box 2 (Sox2) is a transcriptional regulator found in pluripotent cell types, including the multipotent neural stem cells that reside in the SGZ and develop into neurons or glia (Zhang and Cui, 2014). In accordance with our Ki67 labeling, we found that IH₃₀ (n=7) increased the Sox2+ population of cells in the SGZ by 30% when compared to control (n=10) (Figure 4.4A). To better assess whether the neural stem cells were actively expanding under CIH, we examined co-labeling of Sox2 with Ki67 in a subset of experiments. We found that nearly all Ki67+ cells were Sox2+ as well, across both IH₃₀ and normoxia-exposed groups (data not shown). Interestingly, the percentage of Sox2+ cells that were also Ki67+ was increased following IH₃₀ (6.09% control, 9.22% CIH; Figure 4.4B). This data implies that the Sox2+ population of cells is expanded under IH₃₀ and is a large contributor to the increase in SGZ proliferation observed following IH₃₀. Interestingly, when we examined the number of neural progenitor cells under CIH by T-box brain protein 2 (Tbr2) immunolabeling, we found that CIH exposed mice had a greater number of committed neural progenitors (data not shown). When this data is considered alongside the CIH-induced reduction in neurons, it suggests that CIH negatively impacts the morphological and neurochemical development of committed neural progenitor cells into immature neurons (Hodge et al., 2012).

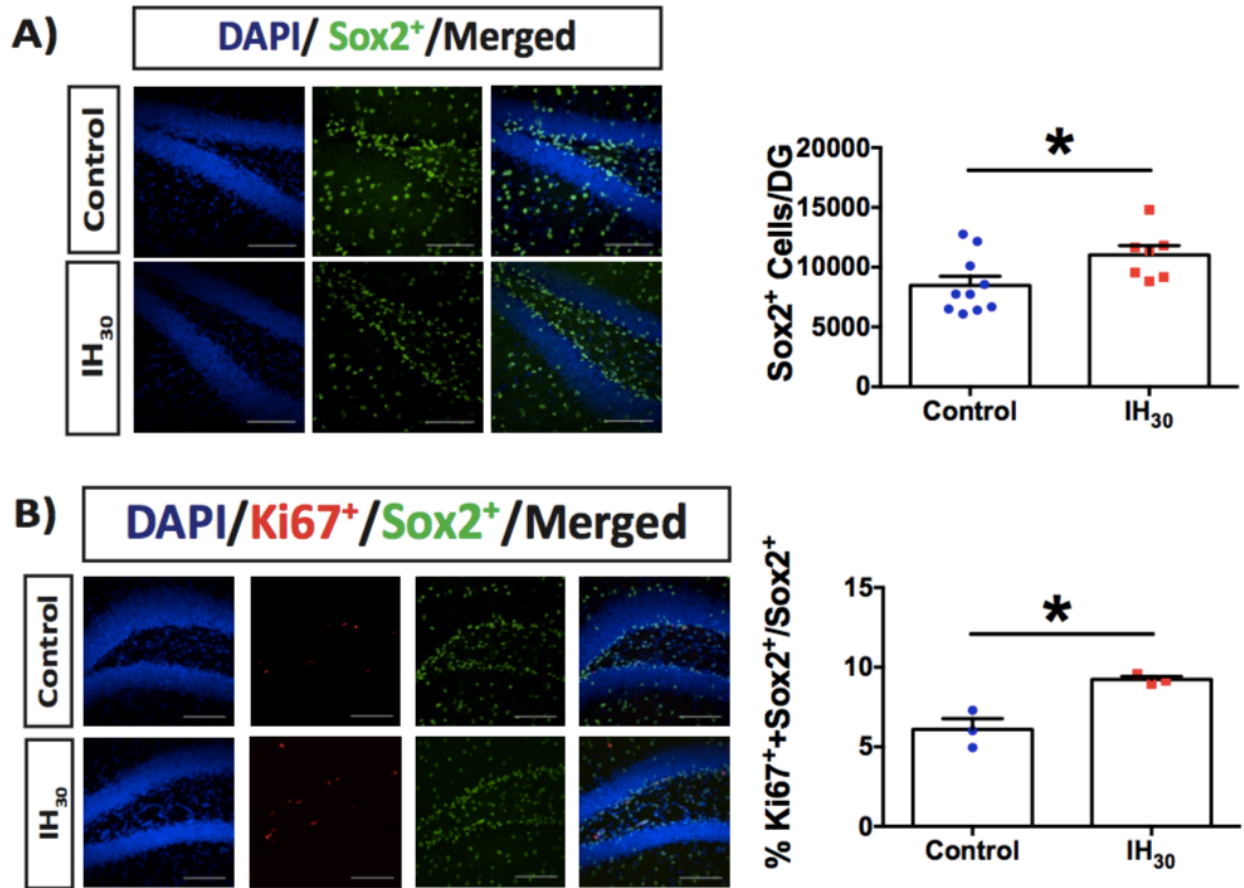


Figure 4.4: IH₃₀ stimulates neural progenitor cell proliferation.

(A) IH₃₀ (n=7) increased Sox2⁺ cells (green) in the SGZ as compared to control (n=10) (p=0.0352). Scale bars: 100μm (B) Double labeled Sox2⁺/Ki67⁺ cells (red+green) showed that a greater proportion of Sox2⁺ cells were actively dividing during IH₃₀ (Control: n=3, IH₃₀: n=3, p=0.0352). Scale bars: 100μm.

Proliferation in the SGZ is important for hippocampal synaptic plasticity and memory formation. Most studies linking neurogenesis and synaptic plasticity demonstrate changes in the number of proliferating cells, either by BrdU or Ki67, along with corresponding changes in LTP. Van Praag demonstrated that exercise increased BrdU along with fEPSP amplitude, LTP, and performance on the Morris water maze (van Praag et al., 1999a). A few studies sought to provide clearer evidence for the role of new-born hippocampal cells by selectively ablating proliferating cells in the hippocampus. As described in Section 4.4, hippocampal irradiation reduced fEPSP amplitude and slope, thus blocking LTP (Snyder et al., 2001; Park et al., 2015). Park and colleagues also demonstrated showed that X-irradiation induced errors in spatial memory discrimination by using a conflict learning shock assessment, thus linking proliferation with hippocampal dependent memory. Increasing the number of newly-born neurons by exposure to an enriched environment is correlated with increased recognition memory, by a longer-lasting preference for a novel object (Bruehl-Jungerman et al., 2005). When proliferation was blocked by an antimetabolic drug, no differences in novel object preference were observed, demonstrating an important role for newly generated cells in hippocampal dependent memory. At first glance, our data appears to be in opposition to these findings, with a reduction in hippocampal dependent memory and LTP paired with an increase in proliferation and number of stem cells. However, the studies discussed above do not focus on the neural stem cell population in isolation, but rather could include neural stem cells, committed neural progenitors, committed glial progenitors. Also, the studies that attempt to follow a single cohort of progenitors, by viral labeling or ablation, have targeted the resulting immature neurons rather than the stem cell population. Previous studies by Gozal

and Pedroso demonstrated increases in proliferation, by Ki67 or BrdU, following CIH (Gozal et al., 2003; Pedroso et al., 2016). Gozal provided evidence that the stem cell population was expanded with an increase in BrdU found in Nestin positive cells, consistent with our data. The lifespan, function, and differentiation of the neural stem cell population under CIH warrant further investigation.

The unique environment of the subgranular niche serves to provide residence and support to the developing hippocampal stem cells of the dentate gyrus. Many SGZ stem cells and neural progenitor cells reside in relative hypoxic zones within the well vascularized dentate gyrus (Studer et al., 2000; Chatzi et al., 2015; Zhang et al., 2015b). Studer and colleagues measured the partial pressure of oxygen within the hippocampus and found that it correlated with a range of 2.6-3.9% oxygen, lower than most tissue perfusion (Ndubuizu and LaManna, 2007). The reduced oxygen content in the dentate gyrus has been demonstrated to promote neurogenesis within the adult rat (Zhang et al., 2015b). These hypoxic niches play an important role in promoting early cell survival and early cell migration to more oxygenated areas within the SGZ leads to a triggering of early apoptosis (Chatzi et al. 2015). Sustained mild hypoxia induces an increase in human neural stem cell proliferation in culture, not unlike what we observed with intermittent hypoxia in the mouse (Santilli et al., 2010). When Studer and colleagues cultured neonatal rat neural stem cells in hypoxic conditions, they also found an increase in proliferation resulting in an increase in total cell number and number of serotonergic neurons, but interestingly, fewer GABA and glutamate neurons, like those found in the dentate gyrus (Studer et al., 2000). Our study is the first to describe the effects of intermittent hypoxia on the adult neural

stem cell population *in vivo*, but leaves us with many questions that could be addressed in a variety of *in vitro* cell culture paradigms.

The neural stem cells and neuroprogenitors of the dentate gyrus are sensitive to changes in the permissive environmental niche of the SGZ (Kempermann et al., 1997; van Praag et al., 1999b, 2005; Song et al., 2012b). NSC proliferation is the highest during youth, and decreases with age (Kuhn et al., 1996; van Praag et al., 2005). Exercise and an enriched environment can attenuate that decline, thus demonstrating a physiological response (Kempermann et al., 1997; van Praag et al., 1999a, 1999b, 2005; Kronenberg et al., 2005). Zhang et al demonstrated an increase in BrdU incorporation in the SGZ following periodic hypoxia similar to several hours at altitude (10,000 feet) (Zhang et al., 2015b).

Paradoxically, NSC proliferation is also observed in pathological conditions, such as social isolation, (Dranovsky et al., 2011; Song et al., 2012a) traumatic brain injury, (Dash et al., 2001; Rola et al., 2006; Barha et al., 2011) stroke, (Jin et al., 2006) Alzheimer's Disease, (Jin et al., 2004; Perry et al., 2012) and Parkinson's Disease (Marxreiter et al., 2013). Our model demonstrates a mild injury to the hippocampus; reduced spatial memory that is found in conjunction with reduced synaptic plasticity and fewer adult-generated neurons (Figures 4.1-4.3). Many brain injury and disease models result in inflammation (Corps et al., 2015; Dzamko et al., 2015; Heneka et al., 2015). After instances of injury or inflammation, low levels of ROS are produced and subsequently activate redox-sensitive signaling pathways that favor cell proliferation (Entman et al., 1991; Floyd and Hensley, 2002; Hensley et al., 2006). Giannakopoulou and colleagues demonstrated that long-term autoimmune CNS inflammation leads to an increase in proliferation within the dentate gyrus. Interestingly, this proliferation did not lead to a greater number of adult-born neurons, but rather an

increase in astrocytes (Giannakopoulou et al., 2017). Prozorovski and colleagues report similar findings when differentiating embryonic mouse cortical progenitor cells under pro-oxidant conditions (Prozorovski et al., 2008). Additional studies show that a model of intermittent hypoxia also leads to the accumulation of reactive oxygen species (Row et al., 2003; Ramanathan et al., 2005; Garcia et al., 2016) and may provide a mechanism by which CIH increases neural stem cell expansion and proliferation.

4.6 MnTMPyP partially mitigates the impact of CIH in the dentate gyrus.

A growing amount of evidence implicates the involvement of reactive oxygen species (ROS) in the mechanism by which CIH disrupts hippocampal physiology (Row et al., 2003; Ramanathan et al., 2005; Kumar et al., 2006; Peng et al., 2006; Garcia et al., 2016). In order to examine whether CIH-induced ROS negatively impacted adult neurogenesis and synaptic plasticity, we treated a cohort of IH₃₀ mice with a concurrent dose of the superoxide anion scavenger, 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)-21H,23H-porphyrin manganese(III) pentachloride (MnTMPyP) (Sharma and Gupta, 2007; Liang et al., 2009b). MnTMPyP is cell-permeable and acts as both a superoxide dismutase and catalase mimetic (Faulkner et al., 1994; Gardner et al., 1996). In order to examine the function of the hippocampal circuit, we again investigated synaptic plasticity in *ex vivo* slices of brain tissue harvested from mice exposed to 30 days of IH with MnTMPyP treatment (IH_{MnTMPyP}). Immediately following the HFS protocol to induce LTP at this synapse, we observed fEPSP potentiation at 130% of baseline (Figure 4.5D middle). The percentage of slope change remained consistent over the course of an hour, consistent with LTP demonstrated in Control animals (Figure 4.5D bottom). When compared with IH₃₀, the

elicited fEPSP immediately following HFS in $IH_{MnTMPyP}$ animals was not significantly different, but the elicited fEPSP at 60 minutes post-HFS was significantly elevated (Supplemental Figure 4.4). This data implicates ROS signaling in the IH-dependent suppression of synaptic plasticity. When the number of newly-born neurons were interrogated by DCX labeling, we found $IH_{MnTMPyP}$ was not significantly different than in Control animals treated with MnTMPyP ($Control_{MnTMPyP}$) (Figure 4.5B). Additionally, $IH_{MnTMPyP}$ yielded many more DCX+ cells than with IH_{30} alone (Supplemental Figure 4.5 top), indicating that MnTMPyP treatment protects against immature neuron loss. Furthermore, in a series of pulse labeling experiments using *nestin-creERT2;Ai27D* mice, we demonstrate no difference in the percentage of adult-born neurons expressing the reporter protein between $CIH_{MnTMPyP}$ and $Control_{MnTMPyP}$ (Figure 4.5C). These data indicate that ROS mediates the IH-induced loss of newly-born neurons in the dentate gyrus. Surprisingly, when we assessed neural progenitor proliferation within the SGZ in $IH_{MnTMPyP}$, we found that the percentage of Sox2+ cells that were also labeled with Ki67+ were significantly greater in $CIH_{MnTMPyP}$ than in $Control_{MnTMPyP}$ (Figure 4.5A). In congruence, no differences were observed in either the total number of Sox2 or ki67 cells in the SGZ of $CIH_{MnTMPyP}$ when compared to IH_{30} alone (Supplemental Figure 4.5 middle, bottom). These findings indicate that neural progenitor cell expansion is due to a ROS-independent mechanism.

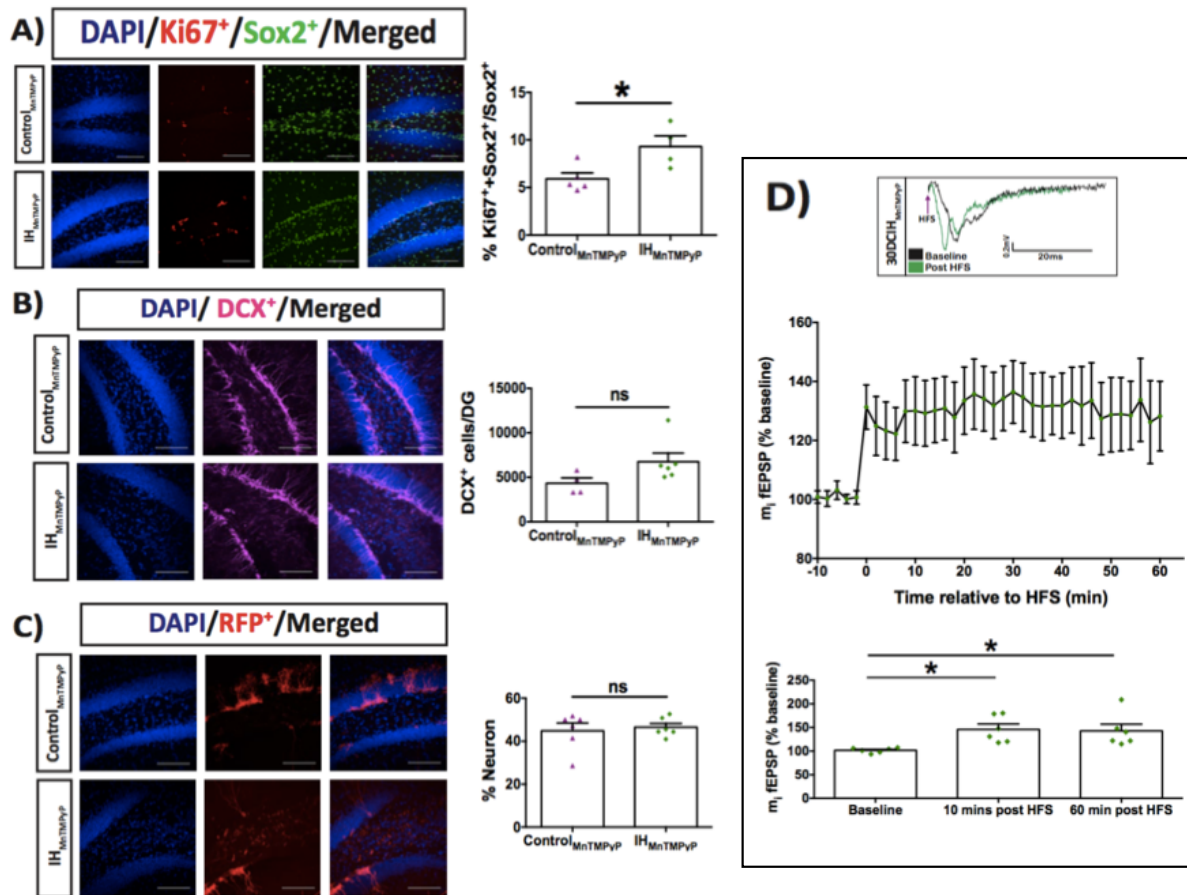


Figure 4.5 MntTMPyP attenuates IH-induced loss of newborn neurons and LTP.

(A) Percentage of Sox2⁺ cells that were Ki67⁺ (red+green) was significantly increased in IH_{MntTMPyP} (n=6) as compared to Control_{MntTMPyP} (n=6) (p=0.0195). (B) IH_{MntTMPyP} (n=6) and Control_{MntTMPyP} (n=4) had similar numbers of DCX⁺ immature neurons (magenta) (p=0.066). (C) IH_{MntTMPyP} (n=6) and Control_{MntTMPyP} (n=6) had similar numbers of RFP⁺ newly-born neurons (red) (p=0.699). (D) LTP was induced in IH_{MntTMPyP} animals. Representative evoked EPSP traces illustrate pre-HFS (black trace) and post-HFS induction (green trace). Post-HFS fEPSP slopes were elevated as compared to baseline at both 10 and 60 mins post-HFS (n=6) Scale bars: 100μm

The ability of MnTMPyP administration during CIH to rescue both synaptic plasticity and the developing neuron population strongly implicates a role for ROS in impairing the neurophysiology of the dentate gyrus and is consistent with previous studies of CIH and ROS. Multiple studies demonstrate ROS is associated with CIH in various tissue types, including brain tissue. Studies by the Gozal group have shown increased protein oxidation, increased lipid peroxidation, and decreased aconitase enzyme activity in the adrenal medulla and carotid body (Peng et al., 2003, 2006; Kumar et al., 2006). Further studies expanded these observations to the brain stem and cerebellum of rodents (Row et al., 2003; Ramanathan et al., 2005; Khan et al., 2011; Garcia et al., 2016). The presence of ROS during CIH is often inferred from experiments utilizing one or more common antioxidants. Again, Gozal and colleagues demonstrate antioxidant reversal of CIH-induced alterations in blood pressure and catecholamine secretion (Kumar et al., 2006; Khan et al., 2011). Phillips showed qualitatively similar data on catecholamine secretion as well as antioxidant mitigated reduction of altered myogenic and vascular responses to CIH (Phillips et al., 2006). When interrogating the effects of antioxidants and CIH in brain tissue, antioxidant treatment mitigated network irregularities within the preBötzinger complex, a region in the brainstem essential for the generation of the respiratory rhythm (Garcia et al., 2016), as well as mitigated deficits in the Morris water maze (Row et al., 2003). Antioxidant administration also abolished protein oxidation observed during CIH as well as normalized HIF1 α mRNA expression (Peng et al., 2006). Our own data of MnTMPyP treatment restoring LTP expression and the number of newborn neurons also implicates oxidative stress as a contributor to CIH-induced hippocampal deficits. Our experiments cannot

discriminate between the occurrence of oxidative stress and the related intracellular signaling response to ROS.

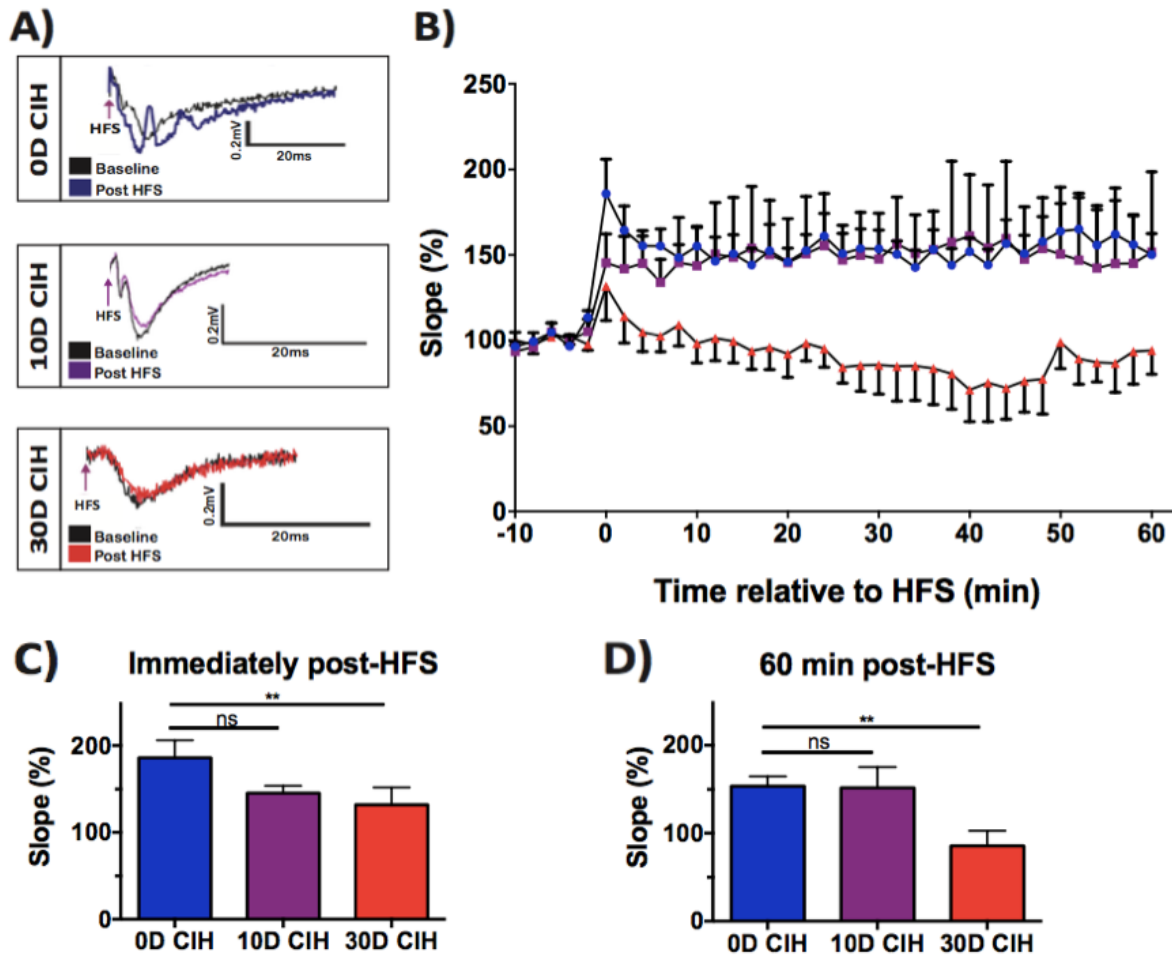
Sustained hypoxia promotes the increase of hypoxia inducible factors, HIF1 and HIF2, to result in a balanced reaction that minimizes the production of ROS. HIF1 α is stabilized by prolyl hydroxylase domain enzymes (PHDs) in the presence of oxygen, and binds with HIF1 β under low oxygen conditions (Semenza, 2000). The HIF1 complex acts as a transcription factor by binding to hypoxia response elements to upregulate greater than 50 gene products integral for developmental and physiological processes, including vascular endothelial growth factor (VEGF) and erythropoietin (EPO) (Semenza and Prabhakar, 2007). HIF1 also induces the upregulation of NADPH oxidase (Nox), a major source of cellular ROS (Bedard and Krause, 2007), and HIF1 α itself, providing positive feedback to maintain the low oxygen signaling. During hypoxia, HIF2 α binds with HIF2 β to upregulate a separate, yet related, set of genes, including several antioxidant enzymes such as Sod1 and Sod2 (Scortegagna et al., 2003). These two complementary proteins minimize the presence of ROS, which is important because severity of ROS determines whether a cell has a physiological or pathological response. For example, nanomolar concentrations of hydrogen peroxide (H₂O₂) promote reversible oxidation of cysteine residues, resulting in protein conformational changes; higher concentrations promote additional oxidation at the same residues, resulting in irreversible protein damage (Schieber and Chandel, 2014). During hypoxia exposure, mitochondria paradoxically generate superoxide radicals, which have been reported to play a role in protein kinase C activation and LTP induction, although the dose of superoxide was not evaluated (Klann et al., 1998; Hamanaka and Chandel, 2010). Kamsler and colleagues showed that low concentrations of H₂O₂ doubled

the fEPSP slope elicited following high frequency stimulation, but high concentrations prevented LTP induction, demonstrating a dose dependent role for ROS (Kamsler and Segal, 2003). Similar to sustained hypoxia, CIH results in an increase in HIF1 α in the adrenal gland as well as in brain tissue (Yuan et al., 2005, 2008; Peng et al., 2006). We did not evaluate the levels of HIF1 α in our study, but our proliferation data is in agreement with a HIF1-induced increase in VEGF and EPO, which have been reported to increase neural stem cell proliferation (Shingo et al., 2001; Licht et al., 2011). In contrast to sustained hypoxia, CIH results in downregulation of HIF2, a reduction in SOD2 transcription, and increased oxidative damage (Row et al., 2003; Ramanathan et al., 2005; Nanduri et al., 2009; Khan et al., 2011; Peng et al., 2011; Garcia et al., 2016). Without the downstream targets of HIF2, CIH may generate an overabundance of ROS that leads to cellular dysfunction, which is supported by our findings that MnTMPyP treatment prevents CIH-induced loss of newborn neurons and failure to induce LTP. Additionally supporting this concept are studies by Peng and colleagues that found that CIH-induced carotid body deficits were reversed or attenuated in either HIF1 α deficient mice or wild-type mice treated with MnTMPyP (Peng et al., 2006). When the cardiorespiratory function of HIF2 α deficient mice was evaluated, a decrease in the transcription of multiple ROS scavengers was associated with increased blood pressure and breathing variability, not unlike the cardiorespiratory dysfunctions observed following CIH exposure (Peng et al., 2011). MnTMPyP treatment also attenuated the cardiorespiratory dysfunction observed in these transgenic mice, similar to the findings of our studies, implying that an imbalance of HIF1 and HIF2 signaling leads to an over-accumulation of ROS that could negatively impact synaptic plasticity and adult neurogenesis in CIH. MnTMPyP treatment did not prevent the

proliferation of Sox2⁺ cells in our studies, suggesting that the severity of ROS influences the early stage of neurogenesis during CIH. In fact, Chatzi and colleagues recently demonstrated that neural stem cells and progenitor cells reside in relatively hypoxic niches within the dentate SGZ, implying that low levels of ROS may be necessary for the maintenance of the proliferative pool (Chatzi et al., 2015). HIF protein imbalance under CIH, and the alterations to downstream effectors, require further investigation.

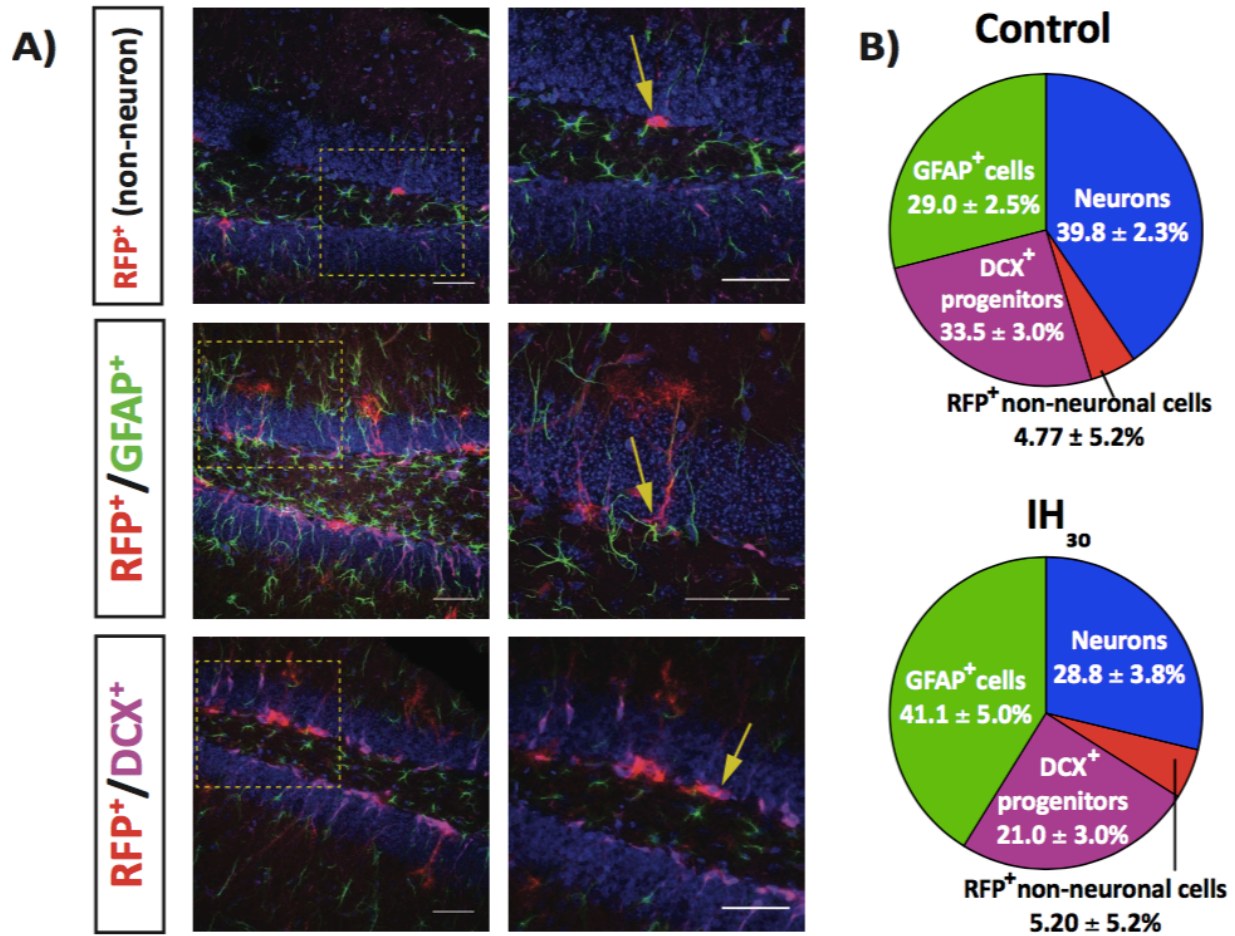
The experiments described in this dissertation demonstrate that chronic intermittent hypoxia negatively impacts multiple aspects of hippocampal neurophysiology. Impaired spatial memory was associated with reduced synaptic plasticity and the loss of newly-born neurons in the dentate gyrus. Antioxidant treatment mitigated the effects of IH on long-term potentiation and newborn neuron number, indicating that ROS plays a role in hippocampal dysfunction observed under IH. Neural progenitor cell proliferation was increased following IH exposure, despite antioxidant treatment, indicating that ROS-independent mechanisms are also involved in IH-mediated changes to the hippocampus.

4.8 Supplemental Figures



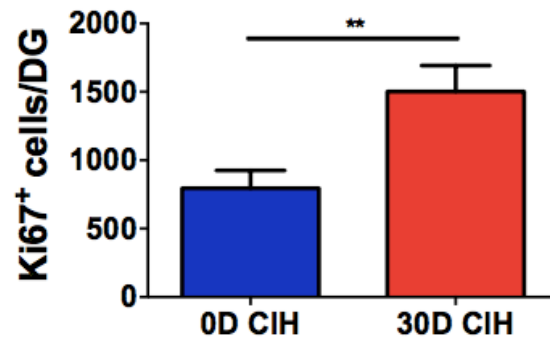
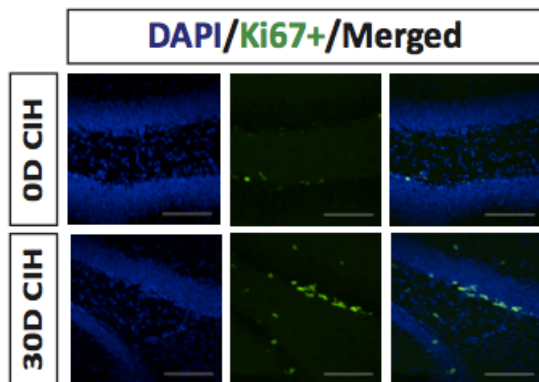
Supplemental Figure 4.1: 10 days of CIH does not affect LTP induction.

(A) Example traces of elicited fEPSP slopes from before (black) and after high frequency stimulation following 0D (blue), 10D (purple), and 30D (red) of CIH. **(B)** Graph represents the percentage change in elicited fEPSP slope from pre-HFS levels. Blue dots are control, purple squares are 10D CIH, and red triangles are 30D CIH. LTP was induced in control and 10D CIH, but not 30D CIH. LTP of the fEPSP following high frequency stimulation (HFS) in 10D CIH was not significantly different immediately post-HFS **(C)** or at 60 minutes post-HFS **(D)**



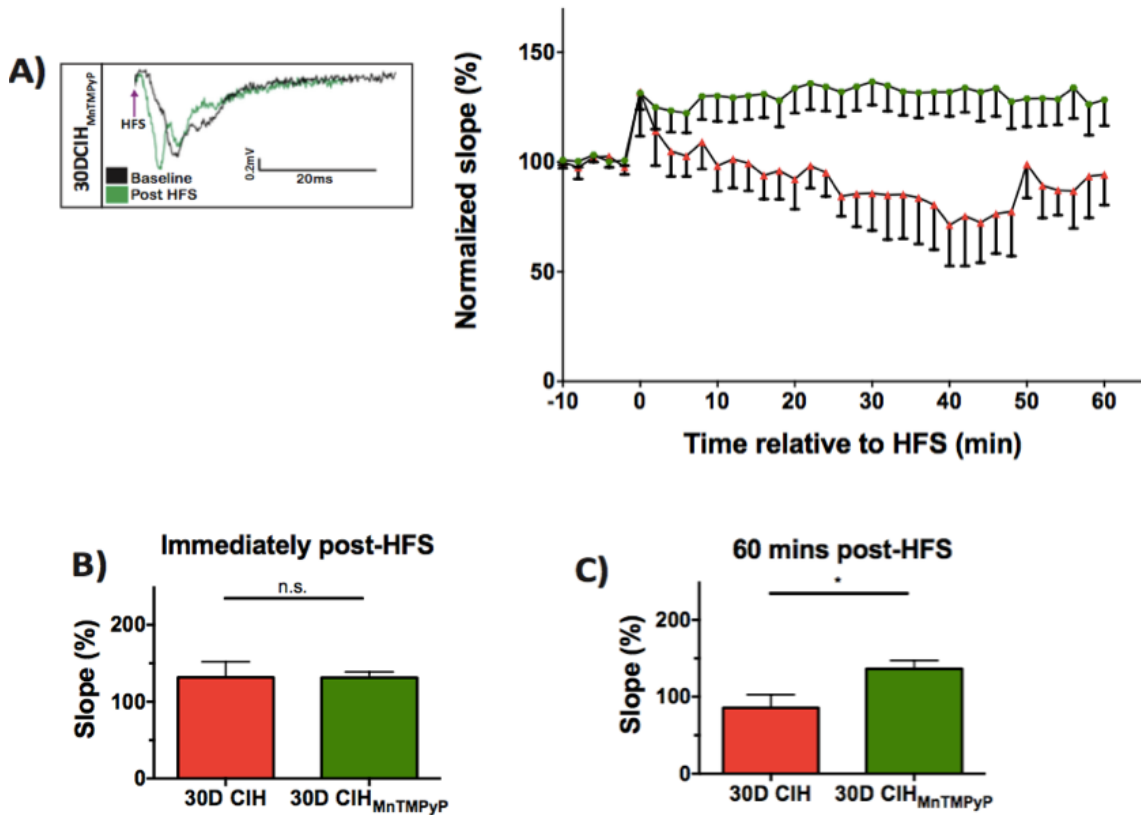
Supplemental Figure 4.2: IH₃₀ affects ratio of neurons to non-neurons.

(A) Birth-labeled cells were split into four distinct categories: RFP⁺ cells that did not express neuronal morphology (top), RFP⁺/GFAP⁺ co-labeled cells (middle), RFP⁺/DCX⁺ co-labeled progenitor cells (bottom) and RFP⁺ cells that exhibit neuronal morphology. Yellow boxes on the left indicate the zoomed-in view on the right-hand side. Yellow arrows indicate example cells of each category. Scale bars: 50 μ m. **(B)** Analysis revealed that following IH₃₀ exposure, the proportion of GFAP⁺ labeled cells is significantly increased ($p=0.0472$) while the proportion of neurons is significantly decreased ($p=0.0267$, shown in Figure 4.3C).



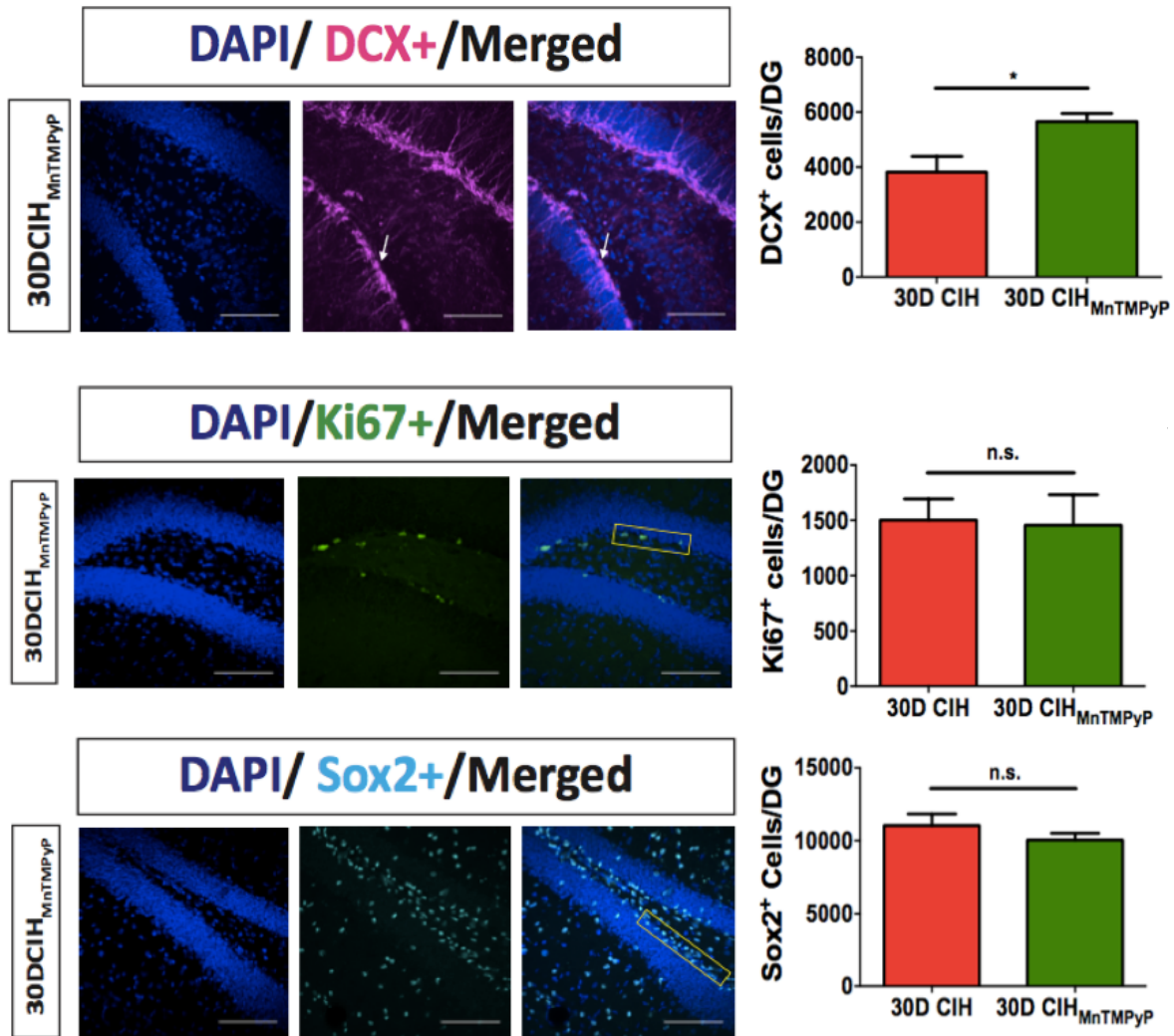
Supplemental Figure 4.3: Intermittent Hypoxia increases proliferation in the subgranular zone.

(A) The number of Ki67-positive cells (green) was significantly elevated after 30D of CIH (n=10) as compared to Control (n=8) (p=0.048). Scale bars: 100 μ m.



Supplemental Figure 4.4: MnTMPyP prevents loss of LTP under IH₃₀.

(A) LTP was established in IH_{MnTMPyP} animals (green circles). IH₃₀ animals are shown in red triangles. Representative evoked EPSP traces illustrate pre-HFS (black) and post-HFS induction (green) (B) LTP of the fEPSP following (HFS) was not different between IH_{MnTMPyP} (n=6) and IH₃₀ (n=6) at 10 mins post-HFS (C) LTP of the fEPSP following (HFS) was not different between IH_{MnTMPyP} (n=6) and IH₃₀ (n=6) at 60 mins post-HFS.



Supplemental Figure 4.5: MnTMPyP prevents loss of newborn neurons under IH₃₀ but does not affect proliferation and neural progenitor number in the SGZ.

IH_{MnTMPyP} (n=6) had significantly greater number of DCX⁺ immature neurons (magenta) than in IH₃₀ (n=5) (p=0.0259). Ki67⁺ cells (green) were not different between IH_{MnTMPyP} (n=14) and IH₃₀ (n=10) (p=0.7888). Sox2⁺ cells (light blue) were also not significantly different in IH_{MnTMPyP} (n=10) as compared to IH₃₀ (n=7) (p=0.4924). Scale bars: 100μm.

Chapter 5

Conclusions and Future Directions

Obstructive sleep apnea is a highly-prevalent respiratory disorder that affects multiple aspects of human physiology (Burwell et al., 1956; Somers et al., 2008). Central nervous system effects include daytime somnolence, fatigue, and mild cognitive impairment (Dempsey et al., 2010; Marshall et al., 2014). Diagnosis and treatment of sleep apnea represents a considerable economic burden for the American population, yet not much is known about the mechanisms by which sleep apnea affects neural physiology (Watson, 2016). The fundamental goal of this dissertation was to test the hypothesis that chronic intermittent hypoxia, a principal consequence of sleep apnea, impairs the cellular based mechanisms of learning and memory in the hippocampus. The major conclusions of this work include: (1) hippocampal function is impaired by chronic intermittent hypoxia (2) adult neurogenesis is differentially affected by chronic intermittent hypoxia and (3) chronic intermittent hypoxia-induced reactive oxygen species negatively impact adult neuronal development and synaptic plasticity in the dentate gyrus. In this chapter, I will summarize the effects of chronic intermittent hypoxia and oxidative stress on hippocampal-based memory, synaptic plasticity, and adult neurogenesis. While discussing the caveats to applying this basic research data to patients with sleep apnea, I will present future experimental directions. Lastly, I will remark on the contributions of work to the

overall field of adult neurogenesis and how it advances translation of our basic studies to possible sleep apnea clinical application.

5.1 Intermittent hypoxia impairs hippocampal function

5.1.1 Behavioral assessments

Our studies demonstrate that chronic intermittent hypoxia results in mild disruption of performance in hippocampal-based memory tasks. CIH-exposed mice performed abnormally in an open field assessment, showed deficits in learning the exit zone of a Barnes maze, and displayed reduced precision in locating the Barnes maze exit zone. However, CIH-exposed mice displayed no deficiency in the recognition of a novel object, thus demonstrating a reduction in various aspects of spatial memory without loss of hippocampal-dependent short-term memory (Moore et al., 2013). Our Barnes Maze data extends previous reports on intermittent hypoxia negatively impacting behavior in the Morris water maze (Gozal et al., 2001). With no forced swim to safety, the rodents experience reduced anxiety and fear in the Barnes maze, revealing a more targeted spatial memory task (Sharma et al., 2010). Indeed, one other report of IH on spatial memory using the Barnes maze shows a qualitatively similar mild deficit (Aubrecht et al., 2015). Anxiety and fear are not completely mitigated in this task, however, as the need to avoid open areas (Barnes Maze table) and seek refuge in small confined areas (exit zone) are helpful instincts for prey animals such as rodents. The difference in open field performance by CIH-exposed mice indicates that anxiety and fear responses may also be affected by intermittent hypoxia. Recent studies have indicated that the ventral region of the dentate gyrus plays a role in mediating anxiety-like behaviors in addition to the more well-known prefrontal regions (O'Leary and Cryan, 2014; Weeden et al., 2015; Schreurs et al., 2017).

Our histological analysis of neurogenesis was limited to the dorsal region of the dentate gyrus, thus preventing the possible correlation between IH-induced anxiety-like behaviors and reduced ventral neurogenesis to be determined. Future experiments should examine these effects, as well as the potential for therapeutic reversal. Our experiments did not include behavioral examinations on animals exposed to MnTMPyP treatment and therefore it remains unknown if reactive oxygen species mediate the mild behavioral effects observed under IH and in patients with sleep apnea. Previous studies have demonstrated that ROS generated by other models of injury can negatively impact performance on hippocampal dependent behavioral tasks, indicating that antioxidant treatment could prove to be a helpful therapeutic for many disorders that result in cognitive decline.

While the hippocampus has been identified as an important contributor to spatial memory, it is neither the sole location of spatial memory in the brain, nor is spatial memory the only aspect of learning and memory that the hippocampus is known for (Ikkai and Curtis, 2011; Yassa and Stark, 2011). Various regions within the frontal and parietal cortices are integral to spatial memory in addition to the hippocampus. As intermittent hypoxia is a global insult, it remains possible that the cognitive deficits observed in patients with sleep apnea are mediated through multiple brain regions including and independent of the hippocampus. Mild reduction in executive function is the most commonly demonstrated deficit amongst sleep apnea patients, and are not easily modeled by rodents. Bizon and colleagues suggest investigating the primarily prefrontal circuits necessary for working memory and cognitive flexibility as stand-ins for executive function, which are behavioral examinations not included within this study (Bizon et al., 2012). The effects of chronic intermittent hypoxia on performance in the delayed alternation radial maze task

and delayed match to sample task would be good extensions of this dissertation work to behaviors more commonly reduced in sleep apnea patients. In addition, future experiments should examine behaviors known to be mediated through additional brain regions to determine whether IH mediates the executive function deficits observed in sleep apnea patients (Kan et al., 2015; Zhang et al., 2016).

5.1.2 Synaptic Plasticity

We evaluated how intermittent hypoxia affects synaptic plasticity in the dentate gyrus by paired pulse facilitation (PPF) and induction of long-term potentiation (LTP). Stimulating the medial perforant pathway and recording from the dendrites of granule neurons of the dentate gyrus, we showed no effects of IH on PPF, but a marked reduction in the ability to induce LTP. Concurrent antioxidant treatment with CIH exposure was able to prevent the loss of LTP induction, albeit with a reduced amplitude than in unexposed control animals. While previous studies on synaptic plasticity following IH have focused on LTP and ROS-sensitivity in the CA1 region of the hippocampus, this study is the first that we are aware of to interrogate PPF in the region and the first to report failure to induce LTP in the dentate gyrus (Payne et al., 2004; Xie et al., 2010). As PPF is primarily associated with enhanced presynaptic neurotransmitter release, normal PPF following CIH indicates that, despite being a global insult, intermittent hypoxia does not induce notable damage to the cells of the entorhinal cortex that project to the dentate gyrus (Zucker, 1989). In contrast, LTP is supported both pre- and post-synaptically, and was found to be suppressed by intermittent hypoxia, indicating that the granule cells of the dentate gyrus may be particularly susceptible to damage in this model (Padamsey and Emptage, 2014). When

Saxe and colleagues selectively ablated newborn neurons in the dentate gyrus, they demonstrated qualitatively similar PPF and LTP data to the work completed in this dissertation, supporting this hypothesis (Saxe et al., 2006). When we interrogated newly-born granule neurons developed under intermittent hypoxia, we found a reduction in the number of cells; the hyperexcitability of young granular neurons allows for these cells to have an outsized contribution to LTP (Schmidt-Hieber et al., 2004; Ge et al., 2007), further supporting the idea that the hippocampal dysfunction induced by sleep apnea is mediated through the loss of newborn neurons.

While our synaptic plasticity data indicates that CIH negatively impacts newborn neurons in the dentate gyrus, we did not evaluate the full electrophysiological characteristics of neurons developed under CIH. Future studies should evaluate several of these characteristics, such as resting membrane potential, spike threshold, and spike amplitude as determined by single-cell electrophysiology of genetically-birth-labeled neurons developed under CIH (Pedroni et al., 2014). Additionally, NMDA receptor activation could be quantified in mice exposed to CIH using whole cell patch clamping, in order to elucidate the observed decay of fEPSPs elicited following HFS. Non-decaying LTP is critically dependent upon increased activation of NMDA-receptors, whose transcription is also necessary for newborn neuron survival (Bailey et al., 1996; Tashiro et al., 2006; Volianskis et al., 2013). In addition, our studies did not evaluate synaptic plasticity in the CA3 region of the hippocampus, where the granule neuron dendrites project and synapse. We demonstrated no gross changes to the volume of the mossy fiber tract, but without knowing whether IH-induced newborn neuron loss affects signaling to CA3, we are missing

an important puzzle piece that could connect sleep apnea and additional hippocampal-dependent cognitive deficits.

5.2 Intermittent Hypoxia Differentially Impacts Adult Neurogenesis

The studies in this dissertation show that chronic intermittent hypoxia reduces the number of adult-born neurons in the dentate gyrus, while simultaneously increasing proliferation of neural progenitors. Describing these data as having a differential impact on adult neurogenesis aids the field by discriminating between active proliferation and the actual survival of neurons in adult neurogenesis. Other researchers have reported that IH induces neurogenesis, but our data following a single cohort of neural progenitors more clearly demonstrate that the opposite is true (Gozal et al., 2003; Pedroso et al., 2016). Proliferation and neurogenesis can be conflated by not pairing neuronal specific markers with proliferation markers. Also, permanently labeling multiple cohorts of developing cells over time can give the appearance of newborn neuronal accumulation, without the proper labeling controls. Here, I will discuss the two distinct effects of intermittent hypoxia on neural progenitor proliferation and adult neuron generation.

5.2.1 IH-induced ROS negatively impacts adult neuronal development

The studies in this dissertation demonstrate that chronic intermittent hypoxia results in the loss of adult-born granule neurons and failure to induce LTP, which were both attenuated by antioxidant treatment. Neuronal apoptosis has been observed in the hippocampus following exposure to sustained hypobaric hypoxia, presumably through glutamate excitotoxicity (Hota et al., 2008; Maiti et al., 2008). Expression of classic hypoxic

response factors, such as HIF1 α , are increased in both hypobaric and intermittent hypoxia, and stimulate oxidative stress (Peng et al., 2006; Malairaman et al., 2014); these pro-oxidant factors are balanced by expression of classic anti-oxidant factors (HIF2) to result in adaptive responses to sustained hypoxia that are simply not observed in intermittent hypoxia (Row et al., 2003; Scortegagna et al., 2003; Nanduri et al., 2009). It may seem counterintuitive that hypoxic injuries would involve reactive oxygen species, but the unbalanced HIF1 α and HIF2 α signals of intermittent hypoxia allows for ROS to be formed and then propagate to a great extent upon tissue reoxygenation; ischemia-reperfusion injury models commonly used in kidney and cardiac research show similar increases in ROS and ROS-mediated damage (Fan et al., 2013; Rodriguez et al., 2013). Sleep apnea and intermittent hypoxia have been shown to be associated with increased oxidative stress, as measured by increased lipid peroxidation, increased protein oxidation, increased NADPH oxidase, and decreased SOD2 expression (Veasey et al., 2004; Peng et al., 2006; Phillips et al., 2006). One of the ways that intermittent hypoxia is distinct from sustained hypoxia is that IH fails to induce an increase in the expression of HIF2 α , the transcription factor that mediates anti-oxidant responses to hypoxia. When we treated our IH-exposed animals with a SOD2 mimetic antioxidant, we found that the loss of both LTP and number of adult born granule neurons were protected. This data indicates that IH-induced ROS negatively impacts adult neurogenesis and synaptic plasticity in the dentate gyrus, potentially providing a mechanism by which sleep apnea induces cognitive decline. What remains unanswered in this dissertation is what stage of neuronal development does IH-induced ROS have its greatest effect? To determine whether ROS impairs late differentiation from committed intermediate neural progenitors or ROS impairs the maturation and survival of

immature adult born neurons, additional experiments must be completed. The Sholl analysis of neuronal dendritic branching that we performed to gain first insight into potential morphological consequences did not reveal any significant abnormalities developed under CIH, suggesting that IH-induced ROS acts earlier in neuronal development. Work completed by Westbrook and colleagues suggests that the transition between committed neural progenitor cell and immature neuron is particularly sensitive to oxidative stress, so interrogating the number and survival of both of these cell types would be beneficial (Chatzi et al., 2015). Preliminary quantification of committed neural progenitor cells by staining for Tbr2 indicates that the same, if not more, cells commit to the neuronal fate under CIH. If these cells are failing to transition to immature neurons, I would hypothesize that they would stain for apoptotic proteins, such as cleaved caspase-3, or would have positive TUNEL labeling of DNA fragmentation. Double immunostaining for these markers with Tbr2 would help determine the stage at which IH negatively impacts adult neurogenesis. Combining birth-labeling with the more sophisticated techniques of single-cell genome and transcriptome analyses could also prove beneficial for determining the molecular differences between cells developed under CIH and normoxia (Macaulay and Voet, 2014; Gawad et al., 2016). Probing for genes associated with hypoxic response, cell cycle regulation, and axonal outgrowth could provide a more detailed snapshot of a cell's response to intermittent hypoxia.

5.2.2 IH induces neural progenitor pool expansion through a non-ROS-mediated mechanism

Although oxidative stress plays a significant role in the development of neurons under chronic intermittent hypoxia, our data indicates that signaling independent of ROS

regulates the pool of neural progenitor cells in the subgranular zone. After IH exposure, we found an increase in the number of proliferating neural progenitor cells which was unresponsive to antioxidant treatment. Elevated proliferation is a common response to brain injury, having been observed after stroke, traumatic brain injury, and Alzheimer's disease (Jin et al., 2004; Villasana et al., 2014; Voitke et al., 2017). Previous studies on intermittent hypoxia also demonstrate an increase in hippocampal proliferation, but our study is the first to demonstrate that ROS does not mediate this effect (Zhu et al., 2010; Zhang et al., 2015b; Pedroso et al., 2016).

It is known that the survival and expansion of neural stem and progenitor cells is regulated in part by oxygenation state (Panchision, 2009; Mazumdar et al., 2010; De Filippis and Delia, 2011; Chatzi et al., 2015). Mild sustained hypoxic conditions have been shown to increase proliferation of neural stem cells in culture, but the role that oxidative stress plays in proliferation has not been addressed (Studer et al., 2000; Santilli et al., 2010; De Filippis and Delia, 2011). Our data demonstrating increased proliferation in the dentate gyrus, with and without antioxidant treatment, indicates that an additional signaling mechanism generated by hypoxia is responsible for progenitor cell expansion. Recent studies demonstrating that oxygen can regulate proliferation in neural stem cells *in vitro* via the Wnt/ β -catenin signaling pathway provide a potential avenue for continued investigation. Mature granule neurons and non-neuronal cells, such as astrocytes, oligodendrocytes, and microglia, influence neural progenitor cell activity via cell-cell contacts and extrinsic signals, including potentially Wnt (Suh et al., 2009; Lavado et al., 2010; Mu et al., 2010; Hodge et al., 2012; Sato, 2015). The effect of Wnt antagonists, with or

without combination antioxidant treatment, on progenitor proliferation, newborn neuron development, and LTP during intermittent hypoxia could help to identify this mechanism.

Interestingly, it hasn't been shown that hypoxia-induced proliferation necessarily yields new neurons *in vivo*, by either previous studies or the work in this dissertation. This begs the question: what happens to these proliferating cells? One potential explanation is a shift away from neurogenesis to favor gliogenesis under hypoxic conditions. The experiments in this dissertation do not support this conclusion, but also do not disprove it either. We show a greater proportion of birth labeled cells co-label with the astrocytic marker, GFAP, post-intermittent hypoxia; however, the cells exhibit morphological characteristics most similar to a type of quiescent neural progenitor cell, which is also known to express GFAP. In light of our Sox2 staining experiments, we believe the increased percentage of GFAP-positive birth labeled cells constitute an increase in neural progenitor cells induced by intermittent hypoxia. More detailed staining analysis should be utilized to demonstrate any change in gliogenesis, including the potential focus on Sox9 labeling outside of the subgranular zone. A second potential explanation is that the hypoxic environment induces increased apoptosis among neural progenitor cells. Again, immunohistochemistry for apoptotic products, activated caspase-3 or TUNEL, in conjunction with known markers of neuronal progenitor cells, such as Sox2, could elucidate whether neural progenitor cells are undergoing apoptosis at a greater rate under intermittent hypoxia.

Independent of mechanism or cell fate, the increased proliferation of neural progenitor cells following intermittent hypoxia appears to be a compensatory-type behavior. Unfortunately, due to the single end-point of our studies, after 30 days of IH

exposure, it is unclear what the progenitor cell expansion could be in response to. It is possible that the cells expand in response to hypoxia, and will continue to proliferate at a high rate throughout chronic exposure, or in response to a loss of a particular cell type known to regulate neurogenesis, such as interneurons or a subtype of neural progenitor cell (Liu et al., 2005; Song et al., 2012b; Kawaguchi et al., 2013). To better understand the expansion of the progenitor pool, a time course study, with both earlier and later endpoints, would be beneficial. Extending exposure of intermittent hypoxia would allow for inquiry into the life span of the neural progenitor pool. Growing evidence suggests that neural progenitor pool expansion is finite, and our progenitor cell proliferation data allows for a potential early extinction of neurogenesis in animals exposed to intermittent hypoxia, regardless of antioxidant treatment (Kippin et al., 2005; Furutachi et al., 2013; Ottone et al., 2014). Additional *in vitro* experiments using neural progenitor cells harvested from animals exposed to intermittent hypoxia could potentially provide insight into the long-term expansion, differentiation capability, and senescence of progenitor cells post-IH exposure.

5.3 Considerations for Future Research

The experiments described in this dissertation are only small steps toward understanding the complexity of neurological response to sleep apnea. There are many avenues that continuing research could investigate, both simple and complex. Necessary analysis of the time course of intermittent hypoxia's effects should be paired with the removal of intermittent hypoxia to interrogate recovery from hypoxia and model what happens to sleep apnea patients who begin CPAP therapy. A comparison of the time course

studies to the physiological adaptation in response to sustained hypobaric hypoxia, like what is experienced when living at altitude, should be undertaken to identify any deficits specifically associated with the pattern of hypoxia exposure. In addition, future experiments could better model the experiences of patients with sleep-disordered breathing.

The studies undertaken in this dissertation were completed using chronic intermittent hypoxia as a model of sleep-disordered breathing, as it mimics the oxygenation pattern experienced by patients with the disorder. Several aspects of sleep disordered breathing that are not accounted for in the model, however. First, the majority of sleep apnea patients begin each period of apnea with complete or partial obstruction of the airway, which involves abnormal pharyngeal reflexes and severe changes to intrapulmonary pressure when breathing effort continues against a closed airway (Remmers et al., 1978; Ramirez et al., 2013). Intermittent hypoxia does not entirely recapitulate apneas nor the abnormal neurological signaling found in the pharynx. Models used by other research groups in rats involve computer-controlled collars or surgically implanted inflatable bags within the trachea to better accommodate these characteristics of obstructive sleep apnea (Farré et al., 2003; Crossland et al., 2013). Transitioning this research to other larger species, such as the rat, would allow for these types of interventions, but reduces the potential use of genetically-modified animals for birth-labeling experiments, a loss that may not be worthwhile to a future researcher. Without additional obstruction interventions, chronic intermittent hypoxia still models many dysautonomic conditions in which sleep-disordered breathing is observed, including Rett syndrome, SIDS, central hypoventilation, and apneas of prematurity. Future experiments

could be tailored to better interrogate the mechanisms of any of these disorders. Secondly, intermittent hypoxia does not cause sleep fragmentation to the extent observed in patients with sleep apnea (Gozal et al., 2001; Ward et al., 2009). As sleep fragmentation is known to induce endothelial cell dysfunction and negatively impact daytime executive function, it remains possible that consistent sleep fragmentation in addition to intermittent hypoxia could exacerbate the effects observed in this dissertation work and should be considered for future studies (Martin et al., 1996; Carreras et al., 2014). Lastly, sleep apnea is more common in patients with additional co-morbidities. Much could be learned from chronic intermittent hypoxia exposure in animals that also exhibit excess weight, eat high-fat diets, or have chronic inflammation. Future researchers should take these caveats into consideration when planning new experiments.

The studies in this dissertation focus exclusively on one particular pattern of hypoxia exposure. The chronic intermittent hypoxia used here was asymmetric, frequent, lasted a third of each day, and extended over a month. We showed deficits in spatial memory, long-term potentiation, and neurogenesis. Often similarly titled as intermittent hypoxia, different patterns of hypoxia exposure are used to mimic working at altitude, usually a mild hypobaric hypoxia that lasts several hours before returning to normoxia and completed over the course of several days (Zhu et al., 2010). Interestingly, this pattern of intermittent hypoxia results increased blood oxygen saturation, increased aerobic capacity, and is used by elite athletes in order to improve athletic performance (Farías et al., 2013). Whether hypoxia results in pathological or physiological effects appears to be dependent upon dosing and schedule. Indeed, low dose, acute intermittent hypoxia has been shown to elicit respiratory motor plasticity, and could potentially restore lost motor function in

diverse clinical disorders that impair motor function (Dale et al., 2014). However, systemic inflammation attenuates these effects, suggesting that inflammatory molecules mediate the cellular response to hypoxia (Huxtable et al., 2013). Our experiments did not assess inflammation systemically or within the dentate gyrus proper, but many other groups have (May and Mehra, 2014). Both sleep apnea and intermittent hypoxia increase systemic levels of inflammatory cytokines (Gozal et al., 2008; Zhang et al., 2015a). These same cytokines are reportedly released from human cardiomyocytes, rodent microglia, and rodent CNS cells following exposure to intermittent hypoxia (Smith et al., 2013; Wu et al., 2016). When brain tissue was interrogated for markers of inflammation following intermittent hypoxia, cytokines that skew microglia to a pro-inflammatory M1 phenotype were found along with activated microglia (Sapin et al., 2015; Snyder et al., 2017). Since microglia are known regulators of adult neurogenesis, particularly in the hippocampus, future experiments should evaluate how inflammation may affect the hippocampal circuit under various exposure lengths and patterns of hypoxia (Sierra et al., 2010; Bachstetter et al., 2011; Reshef et al., 2014; Sato, 2015).

5.4 Summary and Contributions to Medical Research

The studies in this dissertation demonstrate that chronic intermittent hypoxia, a chief characteristic of sleep apnea, negatively impacts the cellular based mechanisms of learning and memory through neurogenesis inhibition. Hippocampal-dependent behaviors were impaired by intermittent hypoxia and correlated with oxidative-stress-dependent deficits in synaptic plasticity and generation of adult-born neurons. Interestingly, intermittent hypoxia also resulted in an expansion of the pool of neural progenitor cells

that was not sensitive to oxygen radical scavenging. Although other groups have interrogated the effects of chronic intermittent hypoxia on hippocampal neurophysiology, these studies are the first to note differential effects on cells at various stages of adult neurogenesis due to this paradigm. The opposing responses to hypoxia and oxygen radical scavenging observed between neural progenitor cells and the neurons they can become reveals an interesting avenue for further research within the field of adult neurogenesis. This work also identifies oxidative stress reduction as a potential therapeutic target for the number of sleep apnea patients without access to consistent, tolerable CPAP treatment. With the aid of future studies, my hope is that the experiments described in this dissertation indicate a tractable path towards protecting hippocampal function in all patients with sleep-disordered breathing.

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Data Attribution

The conceptualization of this thesis project was completed by Chelsea M Pagan (CMP), Alfredo J Garcia III (AJG), and Jan-Marino Ramirez (JMR). Experimental design was completed by CMP, AJG, JMR, and Rebecca D Hodge (RDH). The acquisition and establishment of genetically modified mice was completed by RDH, AJG, CMP and Maggie A Khuu (MAK). The mouse colony was maintained and confirmed by genotyping by CMP and MAK. Exposure to chronic intermittent hypoxia was completed by CMP, MAK, and AJG. Genetic birth-labeling of hippocampal neurons was completed by CMP. Antioxidant treatment was performed by CMP and Seattle Children's Research Institute Office of Animal Care Staff. Behavioral examinations were completed by MAK and analyzed by CMP and MAK. Electrophysiology experiments were conducted and analyzed by MAK and AJG. Tissue processing and histology were conducted by CMP, MAK, RDH, Alexi Z Christakis (AZC), Karissa Lam, (KL) and Emily Parlan (EP). Image collection was completed by CMP, MAK, AZC, KL, EP and RDH. Image quantitation was completed by CMP and MAK. Cell lineage analysis was completed by CMP. Sholl analysis was completed by Thara Nallamothu (TN). CMP and MAK generated the figures used throughout this dissertation. The organization and writing of this dissertation was completed by CMP, with guidance from AJG, MAK, JMR, Robert F Hevner, Kathleen J Millen, and Olivia Bermingham-McDonogh.

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EDUCATION

- 2011-2018 **Ph.D. Pathology - Molecular Basis of Disease Program**
 Howard Hughes Molecular Medicine Certificate
 University of Washington, Seattle, WA
- 2015-2018 Ramirez Lab, Seattle Children's Research Institute
- 2012-2015 Mahoney Lab, UW-South Lake Union
- 2001-2006 **B.S. Biological Sciences – Neurobiology Emphasis**
 Psychology Minor
 University of California, Irvine, CA
-

PROFESSIONAL EXPERIENCE

- 01/15-present **Graduate Research Fellow, University of Washington, Seattle, WA**
 Jan-Marino "Nino" Ramirez, Ph.D.
- Research project: Sleep apnea impairs hippocampal function and adult neurogenesis
 - Technical skills: fluorescent immunohistochemistry; confocal microscopy; animal breeding, husbandry, drug administration, and surgical procedures; statistical analyses
 - Designed and executed multiple experiments to yield first-authored submitted manuscript
 - Disseminated research by poster, print, and oral presentation at internal, departmental, and national conferences
 - Responsible for complete IACUC protocol submission and approval for Ramirez Lab
 - Mentored, trained, and supervised 3 rotational students on experiments of my design
 - Facilitated long-term collaboration on multiple experiments with off-site colleagues
- 07/12-01/15 **Graduate Research Fellow, University of Washington, Seattle, WA**
 William Mahoney III, Ph.D.
- Research project: Potential therapeutic properties of monocytes on vascular remodeling following myocardial infarction.
 - Technical skills: Murine cell culture, transfection, and RNA expression; animal handling, drug administration, and surgical procedures; micro-CT angiography; statistical analyses
 - Designed several animal-use experiments, obtained IACUC approval, executed protocols
 - Coordinated several small collaborative projects with labs within the department, outside of the department, and at independent research institutes
 - Created internal standards of practice for techniques learned during off-site collaboration
 - Disseminated research by poster and oral presentation at internal, departmental, and national conferences
- 03/07-09/11 **Staff Research Associate II, University of California, Irvine, CA**

CDRF SCI Core Laboratory: Aileen Anderson, Ph.D. Director

- Research projects: Effect of thrombate on early inflammatory response following contusion spinal cord injury; synthetic scaffolds as bridges for regeneration following hemisection spinal cord injury
- Temporarily supervised 5 technicians and undergraduates during lab manager's L.O.A.
- Facilitated collaborative projects between the CDRF Core and CDRF Consortium associates, CDRF Pilot Project Grantees, and CDRF Biotech Initiative laboratories
- Coordinated multiple projects to maximize personnel, animal, and surgical resources
- Supervised and performed project planning, rodent survival surgical procedures, animal behavior testing, drug administration, histology, microscopic imaging, data analysis
- Trained internal and external personnel in surgical, behavioral, and histological procedures
- Responsible for equipment and supply purchases and maintenance
- Prepared multiple IACUC protocols and modification for approval

02/08-09/11 **Lecturer, The Berkeley Review, Claremont, Irvine, and Los Angeles, CA
Physiology Department: Todd Bennett, M.S. Chief Executive Officer**

- Prepared and taught lessons on human physiology including, but not limited to, Cardiovascular, Renal, Reproductive, and Immune Systems
- Taught test-taking strategies to students preparing to take the Medical College Admissions Test and to the UC Irvine Post-baccalaureate program

9/04-06/06 **Research Assistant, University of California, Irvine, CA
Laboratory: Aileen Anderson, Ph.D.**

- Research projects: Role of complement proteins in a mouse model of spinal cord injury, Comparison of complement levels in various commonly-used mouse strains
- Using CH50 assays and stereological quantification of injured spinal tissue
- Post-operative daily care of injured research animals

VOLUNTEER EXPERIENCE

03/17-present **HOA Board Secretary, Manhattan Plaza HOA, Seattle, WA**

- Prepare agenda and minutes for open and closed meetings of the Board and HOA
- Evaluate financial, legal, and domestic plans for HOA
- Instruct property management company of all Board decisions
- Correspond with and advise legal team regarding collections for unpaid dues
- Served as one of several on-site managers for emergent housing issues

5/16-11/17 **Bargaining Team Member, UAW 4121, University of Washington
David Parsons, Local UAW 4121 President**

- Facilitated membership and involvement of members in region 5 departments
- Evaluated financial and organizational plans for Local as an executive board member
- Prepared correspondence collaboratively on behalf of the Local for membership-wide communications, University of Washington representatives, and activist organizations
- Aid in the collaborative preparation of bargaining demands and negotiations for employment contracts with representatives of the University of Washington
- Facilitated yearly orientation to new student members in multiple departments

- 09/15-9/17 **G.E.M.S. mentor**
Association for Women in Science, Seattle, WA
Girls in Engineering Math and Science: Jac Fitzgerald, co-chair (2016-17)
- Taught interactive science lessons to JH-aged girls: genetics, neuroscience, engineering
 - Led small team of volunteer instructors: genetics, neuroscience, engineering
 - Instructed hands-on laboratory experiments: chromatography, forensics, computer science
- 1/15-3/17 **Laboratory Assistant, Seattle Children’s Research Institute, Seattle, WA**
Science Adventure Lab: Amanda Jones, Ph.D.
- Led elementary school aged students on educational field trip through SCRI
 - Assisted in age-appropriate lessons in neuroscience, respiration, and career choices
 - Table lead for Guinness Book of World Records “Greatest Number of Simultaneous DNA Extractions”
- 11/14-5/16 **Union Steward, Local UAW 4121, University of Washington**
David Parsons, Local UAW 4121 President
- Facilitated membership and involvement of members in region 5 departments
 - Liaised with members regarding employment contract compliance and insurance coverage
 - Served on the by-laws committee interpreting the language of proposed amendments and advising the membership on potential conflicts with the originating by-laws
 - Facilitated yearly orientation to new student members in multiple departments
- 10/12-10/16 **Scientist Volunteer, Pacific Science Center, Seattle, WA**
Life Science Research Weekend (LSRW): Valerie Kravis
South Lake Union Group: Jill Weyers, PhD
- Hosted a table presenting hands-on study of DNA at yearly LSRW
 - Led primarily elementary school aged students in DNA extraction protocol
 - Coordinated scheduling of additional volunteers and materials acquisition and disposal
-

HONORS AND AWARDS

HHMI/ UW Molecular Medicine Scholar Award, University of Washington 2017, Seattle, WA
Invited Speaker, APS – Respiration, Experimental Biology Conference 2016, San Diego, CA
Recipient of NAVBO travel award for Vasculata 2013, San Diego, CA
Invited Speaker, School of Medicine PhD Welcome, University of Washington, Seattle, WA
Nelson Fausto Graduate Research Presentation Award, University of Washington, Seattle, WA
Dean’s List, University of California, Irvine, CA

SELECTED PUBLICATIONS, POSTERS, AND ABSTRACTS

Khuu MA*, **Pagan CM***, Nallamothu T, Hevner RF, Hodge RD, Ramirez JM, and Garcia AJ III. Intermittent hypoxia-dependent ROS signaling disrupts adult neurogenesis and synaptic plasticity. Journal of Clinical Investigation. Submitted.

Pagan CM, Khuu MA, Christakis AZ, Ramirez JM, and Garcia AJ III. A Murine Model of Obstructive Sleep Apnea Disrupts Synaptic Plasticity and Suppresses Adult Neurogenesis In The Dentate Gyrus. (2017) Keystone Symposia: Neurogenesis during Development and in the Adult Brain, Olympic Valley, CA

Pagan CM, Khuu MA, Christakis AZ, Ramirez JM, and Garcia AJ III. Chronic Intermittent Hypoxia Suppresses Adult Neurogenesis And Disrupts Synaptic Plasticity In The Dentate Gyrus Of The Hippocampus. (2016) Experimental Biology 2016, San Diego, CA

Lundy SD, Gantz JA, **Pagan CM**, Filice D, and Laflamme MA. Pluripotent Stem Cell Derived Cardiomyocytes for Cardiac Repair. Current Treatment Options in Cardiovascular Medicine 2014 Jul;16(7):319.

Pagan CM, Weyers JJ, Minami E, Giannandrea M, Dupras S, Parks WC, Murry CE, and Mahoney WM. Graft vascularization: the role of monocytes in regulating myocardial vascular remodeling. (2013) Vasculata 2013: meeting of the North American Vascular Biology Organization, La Jolla, CA