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Intermittent Hypoxia Alters Metabolic and Cardiovascular Neural Pathways

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INTERMITTENT HYPOXIA ALTERS METABOLIC AND CARDIOVASCULAR NEURAL PATHWAYS

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by

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ABSTRACT

Intermittent hypoxia (IH) is a major pathophysiological manifestation of obstructive sleep apnea (OSA). Previous studies have implicated IH in mediating many pathophysiological outcomes associated with OSA. Only few studies have examined IH-induced alterations to central signaling pathways important in cardiovascular and metabolic phenotypes associated with OSA. This thesis employed a rodent model of IH to examine changes to molecular neural pathways associated with metabolic and cardiovascular pathophysiological outcomes of OSA. Acute IH induces a specific negative body energy balance phenotype. This is concomitant to a reduction in body weight and food intake, with an elevation in food conversion efficiency. Increased plasma leptin concentrations also occur immediately following acute IH, which is mirrored by increased activation of leptin-signaling and satiety-inducing molecules within the arcuate nucleus of the hypothalamus (ARC). The effects observed on body energy balance following acute IH are attenuated in the homozygous leptin-deficient KILO rat, suggesting the importance of elevated leptin in mediating the body energy balance responses following acute IH. Over chronic IH exposure, rats have a complex metabolic phenotype, which includes a reduction in body weight and body fat mass. Throughout the chronic period of exposure, animals develop a resistance to the hormone leptin, the primary hallmark for the development of obesity. This is concomitant to increased food intake and fat-standardized plasma leptin concentrations. Within ARC, leptin-associated signaling pathways are not activated, and there is less protein content of satiety-inducing proteins. There is also more protein of a negative regulator of leptin signaling in ARC following chronic IH.

Chronic IH also elevates resting blood pressure and reduces baroreceptor reflex gain, which are associated with reduced neuroplasticity markers within the nucleus of the solitary tract. Some of the changes observed over acute IH in these markers are leptin-dependent, as they are abolished in the leptin-deficient KILO rat. Leptin appears to interact at the major sensory site for IH, the carotid body, with the renin-angiotensin system, as blockers captopril and losartan inhibit IH-induced alterations to leptin signaling molecules. This thesis shows potential mechanisms by which IH can induce cardiovascular and metabolic phenotypes observed in OSA patients.

KEYWORDS

Intermittent hypoxia

Leptin

Body energy balance

Peripheral chemoreceptor reflex

Neuroplasticity

CO-AUTHORSHIP

Chapters 3 and 5 of this thesis use leptin-deficient KILO rats produced and provided in part by Dr. A McCoy from Sigma Advanced Genetic Engineering Labs (St. Louis, MO, USA).

In Chapter 3, Dr. D. Jones provided the wild-type Sprague-Dawley rats, as well as editorial review of the manuscript.

In Chapter 6 of this thesis, S. Messenger performed some of the western blots in captopril-treated carotid bodies and provided editorial review of the manuscript.

All other experiments conducted as part of this thesis were performed by Jason Moreau under the supervision of Dr. John Ciriello at the University of Western Ontario.

This thesis is dedicated to

Paul Douglas Moreau and *Diane Lynn Moreau*

Your love and support knows no bounds.

I couldn't have done this without you.

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LIST OF ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
ACE	angiotensin I converting enzyme
AgRP	agouti-related peptide
AHI	apnea-hypopnea Index
ANG II	angiotensin II
AP	arterial pressure
ARC	arcuate nucleus of the hypothalamus
AT_1R	angiotensin II type 1 receptor
AT_2R	angiotensin II type 2 receptor
BDNF	brain-derived neurotrophic factor
BMI	body mass index
Capt	captopril
CART	cocaine- and amphetamine-related transcript
CIH	chronic intermittent hypoxia
CPAP	continuous positive airway pressure
CRH	corticotrophin releasing hormone
CSF	cerebrospinal fluid page
ECL	enhanced chemiluminescence
ERK1/2	extracellular signal-regulated kinase 1/2

Fra-1	immediate early gene Fra-1
FRA-1/2	immediate early gene protein products FRA-1/2
GAP-43	growth-associated protein 43
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gp145	long form TrkB neurotropic receptor
gp95	short form TrkB neurotropic receptor
HIF-1	hypoxia-inducible factor 1
HR	heart rate
HRE	hypoxia response element
HRP	horseradish peroxidase
IH	intermittent hypoxia
JAK2	janus kinase 2
KILO	homozygous leptin-deficient rats
Los	losartan
MAPK	mitogen-activated protein kinase
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
ObR	leptin receptor
ObR100	100 kDa short-form leptin receptor

ObRA, C-D, F	short form leptin receptors
ObRB	long form leptin receptor
ObRE	soluble/secreted leptin receptor
OSA	obstructive sleep apnea
PBS	phosphate-buffered saline
pERK1/2	phosphorylated ERK1/2
POMC	pro-opiomelanocortin
pSTAT3	phosphorylated STAT3
PTP1B	protein tyrosine phosphatase 1B
RAS	renin-angiotensin system
ROS	reactive oxygen species
RVLM	rostral ventrolateral medulla
SDS	sodium dodecyl sulfate
sLTF	sensory long-term facilitation
SOCS3	suppressor of cytokine signaling 3
STAT3	signal transducer and activator of transcription 3
TBST	tris-buffered saline + tween-20
TrkB	tropomyosin receptor kinase B
VLM	ventrolateral medulla
WT	wild type control rats

CHAPTER 1

INTRODUCTION AND BACKGROUND

1. INTRODUCTION

This thesis focuses on consequences of intermittent hypoxia, a major pathophysiological manifestation of obstructive sleep apnea. In particular, alterations to energy balance, and the cardiovascular system were assessed. In addition to these physiological changes, associated neural pathways were evaluated for alterations that may serve as determinants in mediating these changes. The first chapter will provide 1) an overview of obstructive sleep apnea, 2) current understanding and mechanisms of hypoxia in body energy balance and cardiovascular disease, and 3) an overview of the leptin hormonal system as it pertains to body energy and cardiovascular systems.

1.1 OBSTRUCTIVE SLEEP APNEA

1.1.1 Disease description

Obstructive sleep apnea (OSA) is a chronic, progressive disease. OSA patients experience episodic partial (apnea) or complete (hypopnea) closure of the upper airway during sleep (Dempsey et al., 2010). These closures prevent appropriate air flow into and out of the lungs, despite respiratory effort. As a result, alveolar partial pressure of oxygen is reduced, leading to a hypoxic state during the airway closure. This, in turn, causes a reduction in blood-oxygen pressure and hypoxemia. The overall effect of these repeated closures during sleep is a state of intermittent hypoxia (IH) and intermittent hypoxemia. These events and their resultant IH lead to arousals, disrupting sleep architecture and

elevating activity of the autonomic nervous system above normal levels. Resulting from the reductions in gas exchange across the blood-gas barrier, hypercapnea is also commonly observed in patients with OSA. Likewise, intrathoracic reductions in pressure are observed as an individual struggles to breathe without success.

The first reports of OSA come from British physicians in the 1870s, who described individuals having "fruitless contractions of the inspiratory and expiratory muscles against glottis obstruction… during sleep" (Lavie, 2003). It was not until the 1960s that OSA was described in obese subjects experiencing arousals resulting from episodic airway obstruction (Gastaut et al., 1966). These results brought to light the concept that obesity, sleepiness, sleep disruption and nocturnal airway obstruction may be linked.

1.1.2 Diagnosis and treatment of OSA

OSA is diagnosed following overnight observation and polysomnography. Determinations of the number of apneic or hypopneic events per hour are calculated, representing the Apnea-Hypopnea Index (AHI). A patient with an AHI greater than five is considered to have a mild form of OSA, while those with an AHI greater than 30 are considered to have severe OSA. Significant reductions blood-oxygen saturation have also been described as important markers for OSA, with a measure of time below 90% blood-oxygen saturation being utilized to identify susceptibility to cardiovascular events (Nieto et al., 2000).

OSA is primarily treated using continuous positive airway pressure (CPAP) devices, since CPAP is highly effective at reducing apneic events and pathophysiology associated with OSA (Faccenda et al., 2001; Jenkinson et al., 1999). That said, there is still a considerable problem with adherence among individuals using CPAP, despite the association between daytime sleepiness and severity of OSA with the likelihood of using the CPAP system (Hoy et al., 1997).

Alternative treatments for OSA include surgery, particularly for individuals with anatomical-related disposition to apnea. The most common surgical intervention for OSA is the uvulopalatopharyngoplasty, which is not necessarily recommended for OSA patients without anatomical dispositions to apnea, since less than half of individuals treated in this way have AHI reduced to less than 20 events per hour.

Oral appliances are another major form of treatment for patients with OSA. The devices used are generally either mandibular advancement devices or tongue retainers, which prevent the collapse of the upper airway (Ferguson et al., 1996, 1997). Certainly these devices have their place in the treatment of mild-to-moderate cases of OSA, and are more effective than surgical interventions, though they are generally less effective than CPAP (American Academy of Sleep Medicine Task Force, 1999).

1.1.3 Prevalence of OSA

The Wisconsin Sleep Cohort, a study involving 602 middle-aged (30-60 years old) subjects, found the proportion of males and females with OSA to be 24% and 9%, respectively. Despite this prevalence, only 4% of males and 2% of females experience daytime somnolence, although having an AHI >5, suggesting a significant under-diagnosis of the disease (Young et al., 1993). More recently, it was estimated that 17% of this same age-group in the United States suffered from at least mild sleep apnea, and approximately 6% suffered from at least moderate sleep apnea, and 41-58% of the burden of sleep apnea was due to excess body weight (Young et al., 2005). Interestingly, countries such as Brazil and some Asian countries have higher prevalence of OSA, despite lower prevalence of obesity (Ip et al., 2001; Sharma and Ahluwalia, 2010). Nonetheless, obesity is a major risk factor for the development of OSA, as an increase of 6 kg/m^2 on the body mass index (BMI) scale results in an increased risk of OSA by 4-times (Young et al., 1993). It is estimated that 70% of OSA patients suffer from obesity (Malhotra and White, 2002).

1.1.4 Comorbidities of OSA

While obesity is a major risk factor for the development of OSA, there are many other comorbidities of OSA. There are three major categories of OSA comorbidities: cardiovascular, metabolic and neurocognitive.

1.1.4.1   Cardiovascular comorbidities

1.1.4.1.1 Systemic Hypertension

Blood pressure in normal individuals is reduced during sleep, compared to normal waking pressures. This "dipping" is not observed in many patients with OSA, attributable to vasoconstriction during the apneic period (Golbin et al., 2008). An event-associated increase in both systolic and diastolic blood pressure was observed in the Sleep Heart Health Study, which involved more than 6000 patients (Nieto et al., 2000). Likewise, Lavie and colleagues (2000) showed a one percent increased risk of hypertension for every increase of one unit on the AHI scale. The Wisconsin Sleep Cohort, a prospective study, determined an event-associated increase in hypertension at four years follow-up, even after accounting for known confounders. Other studies have not displayed a significant effect of OSA on blood pressure, especially when BMI is taken into consideration. However, OSA is considered by many to be a major risk factor for the development of secondary hypertension (Bradley and Floras, 2009; Chobanian et al., 2003). Likewise, use of CPAP is able to reduce blood pressure and sympathetic activity in patients (Dimsdale et al., 2000; Haentjens et al., 2007).

1.1.4.1.2 Pulmonary Hypertension

Pulmonary hypertension is diagnosed following right heart catheterization measurement of pulmonary arterial pressure of greater than 25 mmHg. The pathophysiology of pulmonary hypertension in OSA could be explained by the

fact that chronic respiratory diseases that induce hypoxia appear to remodel the pulmonary arteries (Presberg et al., 2003). Pulmonary hypertension prevalence in patients with OSA has been estimated at 41%, when adjusted for confounding variables, including BMI (Sajkov et al., 1994). CPAP reduces pulmonary arterial blood pressure (Arias et al., 2006).

1.1.4.1.3 Cardiac Arrhythmias

In the cross-sectional Sleep Heart Health Study, severe OSA patients had four times the frequency of having atrial fibrillation, when adjusted for confounders, including BMI. OSA patients had three-times the frequency of having nonsustained ventricular tachycardias when adjusted for confounders (Mehra et al., 2006). Bradyarrhythmias are also found in patients with OSA, and it has been shown that CPAP use can reduce this difference (Guilleminault et al., 1983).

Cardiovascular mortality is elevated in men and women with severe OSA and mortality can be reduced by implementation of CPAP (Marin et al., 2005; Campos-Rodriguez et al., 2012). This may be due to alterations in the timing of severe cardiac events, as it has been shown that these are more likely to occur during sleep hours of patients with OSA, while it is more frequent in the morning for normal people (Gami et al., 2005).

1.1.4.1.4 Stroke

In a prospective study by Redline and colleagues (2010) stemming from the Sleep Heart Health Study, it was shown that moderate-to-severe OSA is associated with a three-fold risk of stroke over an eight year period. This association is supported by findings of the Wisconsin Sleep Cohort, which shows moderate-to-severe OSA is a significant risk factor for stroke (Arzt et al., 2005), and this may be associated with the severity of the disease (Yaggi et al., 2005). Interestingly, the development and progression of OSA may be bidirectionally associated with incident stroke, as apneas are associated with decreased blood flow following stroke, which may induce thrombosis (Netzer et al., 1998). CPAP therapy has beneficial effects in decreasing mortality in OSA patients with stroke (Martinez-Garcia et al., 2009).

1.1.4.2 Metabolic comorbidities

1.1.4.2.1 Obesity

Obesity is an energy balance disease, resulting from a chronic state of positive energy balance, such that energy consumed is not adequately utilized. Excess calories are stored in adipose tissue depots located throughout the body. A concomitant increase in lean mass is also observed, resulting in significant weight gain and elevation of body mass index (BMI; Williams and Fruhbeck, 2009). OSA is associated with obesity, as previously mentioned and illustrated by the fact that obese patients with OSA that undergo bariatric surgery have

improvements in OSA parameters such as oxygen saturation and sleep disturbances (Haines et al., 2007). Similar results have been obtained in studies using sibutramine-induced weight loss (Sutherland et al., 2011). Patients diagnosed with OSA appear to have trouble losing excess weight when compared to non-apneics with the same level of obesity. Likewise, patients with OSA tend to gain weight over the course of their disease (Phillips et al., 1999). As a result, it is clear that a bidirectional relationship exists between obesity and OSA, though this has not received much scientific study.

1.1.4.2.2 Metabolic syndrome

The metabolic syndrome is a cluster of metabolic pathophysiologies that include obesity, insulin resistance, hypertension and dyslipidemia. The criteria by which individuals are diagnosed with the metabolic syndrome include specific cut-offs of specific measures that fall under the umbrella of the pathophysiologies mentioned above, including altered cholesterols, elevated blood pressure and triglycerides, glucose intolerance, and elevated BMI (American Medical Association, 2001). Current guidelines suggest that a patient experience any combination of three of these issues, and obesity is not a requirement for diagnosis. It is estimated that the prevalence of the metabolic syndrome in developed countries is 23% (Ford et al., 2002).

Studies have emerged examining a potential relationship between OSA and the metabolic syndrome. OSA was found to be associated with specific

criteria of the metabolic syndrome, and the syndrome itself, with an odds ratio of around 9 (Coughlin et al., 2004). The severity of OSA may also predict the number of criteria of the metabolic syndrome (Lam et al., 2006). Interestingly, OSA is associated with a worse metabolic profile of individuals who were not obese, and associated with the presence of the metabolic syndrome (Kono et al., 2007).

1.1.4.2.3 Insulin resistance

Insulin resistance is a hallmark manifestation of the metabolic syndrome, and type II diabetes mellitus. The prevalence of OSA within type II diabetics is estimated at 23%, and OSA is correlated with type II diabetes, regardless of appropriate confounders such as BMI, age and neck size (West et al., 2006). The use of CPAP in modulating glycemia has only provided weak, observational evidence to support a role of OSA treatment in improving hyperglycemia (Babu et al., 2005; Hassaballa et al., 2005). The Wisconsin Sleep Cohort failed to show provide an increased incidence of diabetes in OSA patients after adjustment at follow-up, and so a causal role for OSA in diabetes is not able to be made (Reichmuth et al., 2005).

1.1.4.2.4 Dyslipidemia

Many studies have observed associations between OSA and poor lipid profiles. The Sleep Heart Health Study, for example, showed a significant

negative association between OSA severity and high-density lipoprotein levels, whereas a significant positive association was observed between OSA severity and circulating triglyceride concentrations when adjusted for confounders (Newman et al., 2001). Other studies have shown similar effects in patients matched by BMI (McNicholas et al., 2007; Coughlin et al., 2004). Despite this, CPAP intervention studies have failed to show convincing significant improvement in cholesterol or triglyceride levels (Coughlin et al., 2007; Robinson et al., 2004).

1.1.4.3   Neurocognitive comorbidities

One of the primary symptoms of OSA is excessive daytime somnolence. This experience of increased sleepiness is associated with a variety of cognitive deficits including decreased alertness, reduced psychomotor speed and impaired executive function (Beebe et al., 2003). These abnormalities result in increased risk of automobile accidents and a significant reduction in the perceived quality of life (Beebe et al., 2003). A loss of gray matter has been observed in OSA patients, associated with these symptoms, an effect which can be altered by CPAP use (Canessa et al., 2011; Thomas et al., 2012). Interestingly, the use of CPAP appears to have greater beneficial effect on these variables in children, compared with adults (Sanchez et al., 2009; Ferini et al., 2003; Marcus et al., 2012; Gurubhagavatula, 2010).

1.1.5 Intermittent hypoxia as a model for OSA

Animal models employing IH have been used for the study of mechanistic effects of OSA-induced IH since 1992 (Fletcher et al., 1992). There are two major types of rodent IH models: sleep-dependent and sleep-independent IH. Sleep-dependent IH relies on the presence of sleep for the induction of the model and requires significant physiological measurement, including electroencephalography and electromyography (Tagaito et al., 2001). This model is expensive, time-consuming, low-throughput, and rarely employed.

The second, more commonly used IH model is sleep-independent. Reductions in available oxygen and a subsequent return to normoxia are applied to the animal as many as 60 times per hour, with oxygen nadirs ranging from as low as 3% to as high as 10% (Polotsky et al., 2006; Veasey et al., 2004; Gozal et al., 2001; Peng et al., 2003). Specific experimental conditions vary widely between research groups. Exposure to these conditions is typically during the sleep-cycle of the animal, and has been shown to correlate well with oxygen saturation values observed in patients with OSA (Jun et al., 2010; Louis and Punjabi, 2009). Recently, sleep-independent models of IH have been employed (Louis and Punjabi, 2009).

1.2 HYPOXIA-SENSING AND THE CARDIOVASCULAR SYSTEM

1.2.1 Peripheral arterial chemoreceptors

Within the body, hypoxia is primarily sensed by peripheral arterial chemoreceptors. These specialized tissues are made up of type I glomus cells and type II sustentacular cells within the carotid and aortic bodies. These small pieces of tissue are located near the carotid bifurcation and aortic arch. Glomus cells are specialized receptors that are hypoxia-sensitive and release a number of neurotransmitters in response to altered O_2 and CO_2/H^+ homeostasis (Gonzalez et al., 1994). Sustentacular cells are supportive in nature, and don't appear to have a function related to hypoxia-sensing. Changes in altered blood gas homeostasis activate glomus cells, causing the release of excitatory neurotransmitters onto the terminals of the carotid sinus branch of the glossopharyngeal nerve (Gonzalez et al., 1994; Nurse, 2010; Lopez-Barneo, 2003). The subsequent neuronal activity results in activation of cardiorespiratory reflexes within the brainstem to alter blood gases to appropriate levels. Activation of the arterial chemoreceptors initiates what is known as the peripheral chemoreceptor reflex, resulting in an elevation of phrenic motor output to the diaphragm, and both sympathetic and parasympathetic efferent branches of the autonomic nervous system, with a resulting elevation in ventilation and blood pressure, and paradoxical bradycardia (Alsberge et al., 1988; Kumar, 2009).

1.2.2 Chemoreceptor reflex activation of the sympathetic nervous system

Much of the evidence indicating the neural structures underlying the sympathetic responses to peripheral chemoreceptor reflex activation has been acquired in anaesthetized animal preparations. Nonetheless, activation of the peripheral chemoreceptor reflex induces phrenic nerve discharge and sympathetic nerve activity to the heart and blood vessels following specific activation of the carotid bodies or hypoxia (Koshiya et al., 1993; Koshiya and Guyenet, 1996a). This activation is eliminated following denervation of the carotid bodies (Koshiya and Guyenet, 1996a). The sensory component of the carotid chemoreceptor reflex begins with the release of excitatory neurotransmitter onto the afferents of the carotid sinus nerve, resulting in activation of the nucleus of the solitary tract (NTS). The NTS is the primary site of the termination of cardiorespiratory reflexes originating in the aortic and carotid bodies (Ciriello, 1994), and contains neurons that are activated by chemoreceptor stimulation and project to the rostral ventrolateral medulla (RVLM; Koshiya and Guyenet, 1996b; Ciriello and Moreau, 2013). It is believed these NTS-RVLM neurons are independent of baroreceptor input and respiratory entrainment, regardless of the activity of the chemoreceptor reflex (Koshiya and Guyenet, 1996b). The sympathetic outflow from the activation of the chemoreceptor reflex originates as signals from the pre-sympathetic neurons of the RVLM, perhaps due to activation of these NTS-RVLM neurons. The recruitment of a given subset of these neurons will alter the tissue to which sympathetic tone will be changed by activating specific sympathetic pre-ganglionic neurons of the spinal cord (Dampney, 1994).

How these neurons are recruited to mediate an elevation in sympathetic tone is still a matter of controversy (Guyenet, 2000). Increases in hypoxia-related sympathetic activity will cause vasoconstriction (Hudson et al., 2011) and a release of catecholamines from the adrenal medulla (Prabhakar et al., 2012).

1.2.3 Intermittent hypoxia and cardiorespiratory reflexes

1.2.3.1   Effect on peripheral chemoreceptor reflex

Individual bouts of short-term hypoxia are observed during IH, which activate the peripheral chemoreceptor reflex in the same way as continuous hypoxia mentioned above, though with a reduced ventilatory response (Prabhakar et al., 2005; Reeves et al., 2003). In fact, a potentiation of the chemoreceptor reflex due to sensory long-term facilitation (sLTF) of the chemoreceptors has been identified in animals exposed to IH (Prabhakar et al., 2005; Prabhakar et al., 2009), but not sustained hypoxia (Baker and Mitchell, 1999; 2000). Likewise, acute IH alters expression of glutamatergic receptors within the dorsocaudal brainstem (containing the NTS region), whereas sustained hypoxia does not (Reeves et al., 2003). This sLTF is concomitant to an elevation of sympathetic nervous tone and blood pressure following acute IH in both rats (Dick et al., 2007; Xing and Pilowsky, 2010; Mandel and Schreihofer, 2009) and humans (Leuenberger et al., 2005). Even after acute exposure to IH, this increased sympathetic activity persists following the discontinuation of

hypoxia (Fuller et al., 2000), though the increased blood pressure was transient (Leuenberger et al., 2005).

Chronic IH (CIH), like acute IH, has significant cardiovascular consequences. Long-lasting elevations in resting blood pressure and sympathetic activity are found in animals (Zoccal et al., 2007, 2008; Bao et al., 1997) and humans (Tamisier et al., 2011) following CIH. These changes in blood pressure are likely mediated by a combination of the sympathetic nervous system (Fletcher et al., 1992b), and vascular factors such as angiotensin II (Ang II), catecholamines and vasopressin (Foster et al., 2010; Bao et al., 1997; Fletcher et al., 1999; Fernandes et al., 2005).

The role of the peripheral chemoreceptor reflex in mediating the CIH-induced blood pressure effects are highlighted by the fact that denervation of the carotid sinus nerve prior to exposure to CIH in rats, eliminates the cardiovascular alterations observed (Fletcher et al., 1992a). Meanwhile, the autonomic alterations observed following hypoxia are eliminated when chemoreceptors are inhibited by hyperoxia (Querido et al., 2010).

CIH potentiates the sympathetic response to additional bouts of hypoxia. In studies assessing the afferent signaling from the carotid bodies, previous exposure to CIH, but not normoxia, caused an increased cellular response (Peng and Prabhakar, 2004; Rey et al., 2004). A similar effect was observed for efferent sympathetic responses to hypoxia (Huang et al., 2009; Greenberg et al., 1999).

At least some of this response is mediated by an alteration in central responses within NTS (Kline, 2010).

1.2.3.2   Effect on arterial baroreceptor reflex

In opposition to the peripheral chemoreceptor reflex, activation of the arterial baroreceptor reflex causes a reduction in sympathetic tone and blood pressure. The baroreceptors are located within the walls of the vasculature of the carotid sinus and the aortic arch and send neural projections into the NTS (Ciriello, 1983; Davies and Kalla, 1981), which projects to the caudal VLM. Activation of the baroreceptor reflex causes an inhibition of the RVLM via monosynaptic connections from the caudal VLM (Agarwal and Calaresu, 1991; Agarwal et al., 1990).

During IH, the baroreceptor reflex would activate to buffer the elevation in blood pressure. In animals with experimental peripheral chemoreceptor activation using potassium cyanide, baroreceptors become activated following the initial rise in blood pressure, resulting in a biphasic response that includes subsequent hypotension and bradycardia (Braga et al., 2008).

Given that animals and humans exposed to CIH develop hypertension, it may be concluded that the baroreceptor reflex is inhibited or desensitized following long-term IH exposure. In the short-term, IH resets the baroreceptor reflex to operate at higher levels of blood pressure and sympathetic tone, not changing the sensitivity of the reflex (Monahan et al., 2006), independent of

changes to ventilatory responses (Halliwill et al., 2003). However, following CIH, desensitization of the baroreceptor reflex may also occur (Gu et al., 2007; Lai et al., 2006).

1.3 HYPOXIA AND BODY ENERGY BALANCE

Hypoxia induces, above all else, a state of negative energy balance. This occurs initially at the level of the mitochondria, reducing the capacity for oxidative phosphorylation, and thus production of ATP. The response to this reduction in energy production is manifested by the release of macromolecules from appropriate storage sites, likely mediated by the sympathetic nervous system, resulting in a reduction in body weight.

1.3.1 Cellular energy responses to hypoxia

Cells are sensitive to alterations in available oxygen concentrations within their environment. Given the role of oxygen in energy production through mitochondrial oxidative phosphorylation, it is not surprising that cells are able to alter both the supply and demand of oxygen within their interstitium through various mechanisms including augmented glycolytic and reduced oxidative phosphorylation pathways.

1.3.1.1 Hypoxia-inducible factor 1

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric protein made up of α and β protein subunits. HIF-1β is a constitutively expressed protein that does not have activity independent of HIF-1α and isn't affected by oxygen tension. In contrast, HIF-1α is the primary activator of the HIF-1 complex (Wang et al., 1995). HIF-1α has domains for binding to HIF-1β, an oxygen-sensitive core and a DNA-binding domain for regulation of transcriptional promoters or enhancers containing a hypoxia-responsive element (Jiang et al., 1997). During normoxia, oxygen binds to the oxygen-sensitive core, resulting in hydroxylation, ubiquitination and subsequent degradation of HIF-1α. During hypoxia, there is less oxygen to bind to the oxygen-sensitive core, and HIF-1α is free to translocate to the nucleus to associate with HIF-1β, forming HIF-1. The heterodimer HIF-1 binds to regulators of genes, which are deemed to be hypoxia-responsive (Chandel, 2010).

1.3.1.2 Generation of ATP

During hypoxic insults, mitochondria reduce their uptake of oxygen thereby limiting their production of ATP, and increasing anaerobic ATP production. This is done to avoid forming anoxic cellular conditions, which may ultimately lead to cell death.

HIF-1 acts as a "switch" to augment the translocation and activity of glucose transporters in the cell membrane, increasing the influx of glucose for the

use in so-called fermentative pathways (Gleadle et al., 1997; Maxwell et al., 1997). The production of ATP by these pathways is oxygen-independent and less efficient at producing ATP than oxidative phosphorylation. Despite this, cells can maintain glycolytic production of ATP at nearly the same level due to an elevated capacity for glucose transport and an increase in glycolytic enzymes, mediated by HIF-1 (Gleadle et al., 1997; Maxwell et al., 1997; Mathupala et al., 2001; Semenza et al., 1994, 1996).

The shunting of pyruvate and other necessary co-factors of oxidative phosphorylation away from the mitochondria and into glycolytic pathways prevents an elevation in reactive oxygen species associated with inefficient electron transport chain activity (Weideman and Johnson, 2008). The enzymes important in these effects are regulated by HIF-1 (Kim et al., 2006; Papandreou et al., 2006).

Reactive oxygen species production has also been shown to occur following the reoxygenation events observed in IH. The increase in available oxygen within tissues following a return to normoxia not only provides the substrate for the production of reactive oxygen species, but also may alter the antioxidant capacity of cells (Singh et al., 2001), leading to lipid peroxidation, formation of reactive nitrogen species and oxidative stress (Row et al., 2003; Xu et al., 2004)

1.3.2 Organism energy responses to hypoxia

1.3.2.1   Body weight and food intake

It has been largely reported that hypoxia of both a continuous and intermittent nature causes an acute reduction in body weight (Westerterp-Plantenga et al., 1999; Martinez et al., 2008). Interestingly, this effect in sustained hypoxia is mirrored by a reduction in food intake, when locomotion, stress and temperature are taken into consideration. Alterations in food intake were concomitant with an increase in the number of meals taken per day. Subjects of this study indicated they felt hungry, but did not have the drive to consume food, an effect observed during altitude sickness. This imbalance in body energetics is currently without a potential mechanism, but has been suggested to be the result of elevated plasma leptin concentrations (Tschop et al., 1998). Ratios of the consumption of specific macronutrients do not appear to be affected by chronic hypoxia (Westerterp et al., 1992; Westerterp et al., 1994; Westerterp et al., 1996). Currently, there are no studies examining food consumption and body energy balance during intermittent hypoxic exposure, though losses in body weight have been reported (Martinez et al., 2008; Ling et al., 2008; Drager et al., 2011).

1.3.2.2   Lipid metabolism

In normal and atherosclerosis-prone animals, chronic IH increases total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein and triglycerides, effects which are amplified by the level of the hypoxic stimulus (Jun

et al., 2010; Li et al., 2005; Li et al., 2007). In support of these findings, increased lipid synthesis pathways of the liver have been shown to be altered (Li et al., 2005; Li et al., 2007; Savransky et al., 2007; Li et al., 2007), an effect which mimics acute exposure to hypoxia (Piguet et al., 2009). Likewise, this effect may be mediated partially by HIF-1 (Li et al., 2006).

In addition to elevated production of lipids, IH has been shown to induce lipolysis, releasing free fatty acids from adipose tissue, likely through activation of the sympathetic nervous system (Jun et al., 2010; Jaworski et al., 2007; Lafontan and Langin, 2009; Zechner et al., 2009). Chronic IH has been shown to induce liver enzymes and liver injury in rodents (Savransky et al., 2007), as well as cause the release of pro-inflammatory cytokines (Savransky et al., 2007).

1.3.2.3   Glucose metabolism

IH is capable of producing an acute insulin resistance in lean mice, and potentiating that found within obese leptin-deficient mice (Iiyori et al., 2007; Polotsky et al., 2003). As well, in human IH models, these effects have been observed without increasing the circulating level of leptin (Louis and Punjabi, 2009). These effects could be mediated by changes in lipid production within the liver, or by increased sympathetic tone leading to lipolysis and ultimately an increase in free fatty acids. Free fatty acids have been shown to reduce glucose uptake by inhibition of insulin pathways within skeletal muscle (Delarue and Magnan, 2007; Kim et al., 2001). Meanwhile, glycogen is released from, and

glucose uptake is reduced, in muscle. Elevated hepatic gluconeogenesis and hyposecretion of insulin may also be mediated by the sympathetic nervous activation following IH. Additional mechanisms mediating insulin resistance in IH include inflammation (Cai et al., 2005; Yuan et al., 2001) and corticosteroid release (Yokoe et al., 2008; Morton, 2010).

1.4 LEPTIN SIGNALING

1.4.1 Leptin and its receptors

The hormone leptin is produced from the *ob* gene, located on chromosome 6 of mice and chromosome 7 in humans. The genes are 84% homologous, containing 3 exons. Leptin is produced as a non-glycosylated protein made up of 167 amino acids, including a 21 amino acid signal peptide (Auwerx and Staels, 1998). The protein contains 4-alpha helices, each of which is 5-6 turns long, connected by lengthy crossover linkages, similar to cytokines like ciliary neurotrophic factor and leukemia inhibitory factor. A single disulphide bridge exists between cysteines 96 and 146, which appears to be crucial for the proper folding and receptor binding of leptin (Zhang et al., 1997). Two major types of mutations occur, which affect the *ob* gene: a non-sense mutation producing a premature stop codon; and a mutation within the gene promoter, inhibiting transcription altogether (Halaas et al., 1995).

Leptin exerts its physiological responses through the leptin receptor (ObR). ObR contains a single transmembrane spanning region, and is located on

chromosome 1 in humans. The receptor protein belongs to the class-1 cytokine receptor family (Tartaglia et al., 1995). ObR can be alternatively spliced to form several receptor isoforms (ObRA-F), which share common extracellular and transmembrane domains, though a variable intracellular domains. These receptors can be classified as short-form (ObRA, C-D, and F), long-form (ObRB), or soluble/secreted (ObRE) (Wang et al., 1998; Lee et al., 1996; Tartaglia et al., 1995). The long-form, ObRB, contains intracellular motifs for the binding and activation of janus kinases (JAK) and signal transducers and activators of transcription (STAT; the so-called JAK/STAT pathway will be discussed in section 1.4.4).

The various isoforms of ObR have unique roles in leptin signaling and secretion. ObRB mediates most of the intracellular signaling associated with leptin binding to the extracellular motifs. ObRA, containing a short intracellular motif, is important for the movement of leptin across membrane barriers, including the blood-brain barrier and from the cerebrospinal fluid into brain parenchyma (Tartaglia et al., 1995; Mercer et al., 1996; Fei et al., 1997; Bjorbaek et al., 1998; Hileman et al., 2000). The ObRE soluble leptin receptor is important for modulation of circulating leptin and its secretion from adipocytes (Ge et al., 2002; Huang et al., 2001; Lammer et al., 2001; Yang et al., 2004).

1.4.2 Regulation of leptin secretion

The hormone leptin is produced by many tissues in the body, including adipose tissue, heart, carotid bodies, brain and gonads. Primarily, white adipose tissue is the major site of production of leptin. White adipose tissue is found

throughout the body in major depots, including the visceral, subcutaneous, retroperitoneal and epididymal fat pads. Each fat pad releases different amounts of leptin, with the epididymal pad releasing the most (approximately 10 ng/10 million cells) in rats. In humans, the subcutaneous fat pad releases the most leptin (Zheng et al., 1996; Arner, 2001). The average circulating level of leptin in normal adults is 3-5 ng/ml, though this value can be much higher (8-90 ng/ml) in obese individuals (Shek et al., 1998; Klein et al., 2004). Initially, it was held that leptin secretion was proportional to the level of white adipose tissue within an individual. The white adipose tissue produces, stores and secretes leptin. The amount of leptin from rough endoplasmic reticulum to golgi to secretory vesicles of adipocytes increases, suggestive of a concentration of leptin prior to its release. Basally, leptin is produced and secreted into the interstitium of adipose tissue to maintain a set leptin concentration within and outside of the adipocyte (Cammisotto and Bendayan, 2007).

Leptin secretion is strongly linked to circadian rhythm: its plasma concentration is lowest in the morning and highest in the middle of the night (Licinio et al., 1997; Mastronardi et al., 2000; Nagatani et al., 2000). Post-prandial elevations in insulin have also been described as an important factor for leptin secretion, though this occurs long after mealtime, suggestive of a long-term, not short-term role of leptin in energy balance (Koopmans et al., 1998; Lynch et al., 2006).

The stimulation of leptin secretion from the adipose appears to be calcium-independent (Cammisotto and Bukowiecki, 2004), though leptin

synthesis is dependent on the presence of glucose (Whitehead et al., 2001). This effect is likely mediated by alterations in ATP (Mizuno et al., 1996; Mueller et al., 1998). Insulin, within the physiological range, is sufficient to increase the secretion of leptin, which is potentiated by the presence of amino acids such as leucine (Cammisotto and Bukowiecki, 2002; Cammisotto et al., 2005; Lynch et al., 2006). The post-prandial insulinemic response, and subsequent glucose-dependent induction of ATP may explain the rise in adipocyte ATP following food consumption (Thompson, 1996; Lynch et al., 2006). Glucocorticoids have been shown to increase leptin release from adipose tissue, likely through alterations in transcription of the protein (De Vos et al., 1995; Bradley and Cheatham, 1999).

Intriguingly, plasma fatty acids do not alter leptin production or secretion, though lipolysis significantly attenuates leptin release (Cammisotto et al., 2003). This is likely due to the role of catecholamines in lipolysis, as exercise, cold exposure and adrenergic stimulation all inhibit leptin secretion, and are lipolytic events associated with cyclic adenosine monophosphate signaling (Bramlett et al., 1999; Rayner and Trayhurn, 2001; Gettys et al., 1996).

Hypoxia is also an important regulator of leptin secretion. Recently, a hypoxia-response element (HRE) was discovered in the 5' flanking region of the human *ob* gene. It was also shown that leptin transcription was increased following hypoxic exposure (Ambrosini et al., 2002). This increase in leptin transcription was induced by HIF-1α/β binding to the HRE domain of the leptin promoter (Ambrosini et al., 2002; Grosfeld et al., 2002).

1.4.3 Leptin-related signaling pathways

Given that ObR is homologous to class I cytokine receptors, it is likely that ObR activity will resemble that of other cytokines. Like other cytokine receptors, ObRB activates JAK/STAT pathways. Upon leptin binding, JAK2 autophosphorylates ObRB on specific tyrosine residues (Kloek et al., 2002; White et al., 1997; Banks et al., 2000). When phosphorylated at Tyr_{1138}, ObRB recruits signal transducer and activator of transcription 3 (STAT3), resulting in phosphorylation of tyrosines on STAT3, resulting in homodimerization and translocation of pSTAT3 to the nucleus (White et al., 1997; Banks et al., 2000; Vaisse et al., 1996). Activated STAT3 acts as a transcription factor, and can activate the transcriptional activity of genes associated with leptin signaling including pro-opiomelanocortin (POMC), immediate early genes and suppressor of cytokine signaling 3 (SOCS3) (Banks et al., 2000; Munzberg et al., 2003; Bates et al., 2003). In addition to the activation of the JAK2/STAT3 pathways, extracellular signal-regulated kinase 1/2 is also phosphorylated, though this may be done directly by JAK2, instead of ObRB (Banks et al., 2000) SOCS3 acts as an important negative feedback regulator of ObRB activation, inhibiting the phosphorylation of JAK2, and thus ObRB and extracellular signal-related kinase 1/2 (ERK1/2). SOCS3 may accumulate following the chronic activation of ObRB (Anubhuti and Arora, 2008). Protein tyrosine phosphatase 1B (PTP1B) is also an important regulator of leptin receptor activity, and has been shown to inhibit JAK2 phosphorylation (Sahu, 2004; Bjorbaeck and Kahn, 2004; Zabolotny et al., 2002; Kaszubska et al., 2002; Cook and Unger, 2002; Figure 1.1). Interestingly, some

have shown that ObRA has the capacity to phosphorylate JAK2, though it is unable to induce STAT3 activation (given that the crucial phospho-Tyr1138 is absent in ObRA), suggestive of a potential activation of ERK1/2 by ObRA (Bjorbaek et al., 1997; Ghilardi et al., 1996).

1.4.4 Leptin and energy balance

1.4.4.1 Food intake

Leptin has many sites of action throughout the body, though the brain is the primary site for its major physiological effect to reduce food intake. Hypothalamic sites of leptin action have drawn particular interest from the scientific community, primary of which is the arcuate nucleus of the hypothalamus (ARC). Within this region exist specific populations of neurons that respond to leptin and contain ObRB. Neuronal cell bodies expressing POMC are found within this region (Elias et al., 1998; Korner et al., 1999). POMC is cleaved within these neurons by prohormone convertases to produce alternative cleavage products that may regulate the autonomic and anorectic effects of leptin, including the production of α-melanocyte stimulating hormone (α-MSH). By acting through the

Figure 1.1 Relevant leptin signaling pathways.

Graphical representation of signaling pathways associated with the binding of leptin to the long-form (ObRB) of its receptor. Upon leptin binding, JAK2 becomes phosphorylated, resulting in the phosphorylation of ObRB at Tyr1138, which causes the phosphorylation and homodimerization of STAT3. pSTAT3 translocates to the nucleus to regulate the transcription of genes and ultimately their protein products including immediate early gene product Fra-1/2, POMC and SOCS3. SOCS3 will cause inhibition of the activation of the leptin receptor through inhibition of JAK2, which can also be regulated by PTP1B. ERK1/2 may also be phosphorylated by activation of ObRB. Arrows indicate activation, blunted lines indicate inhibition.
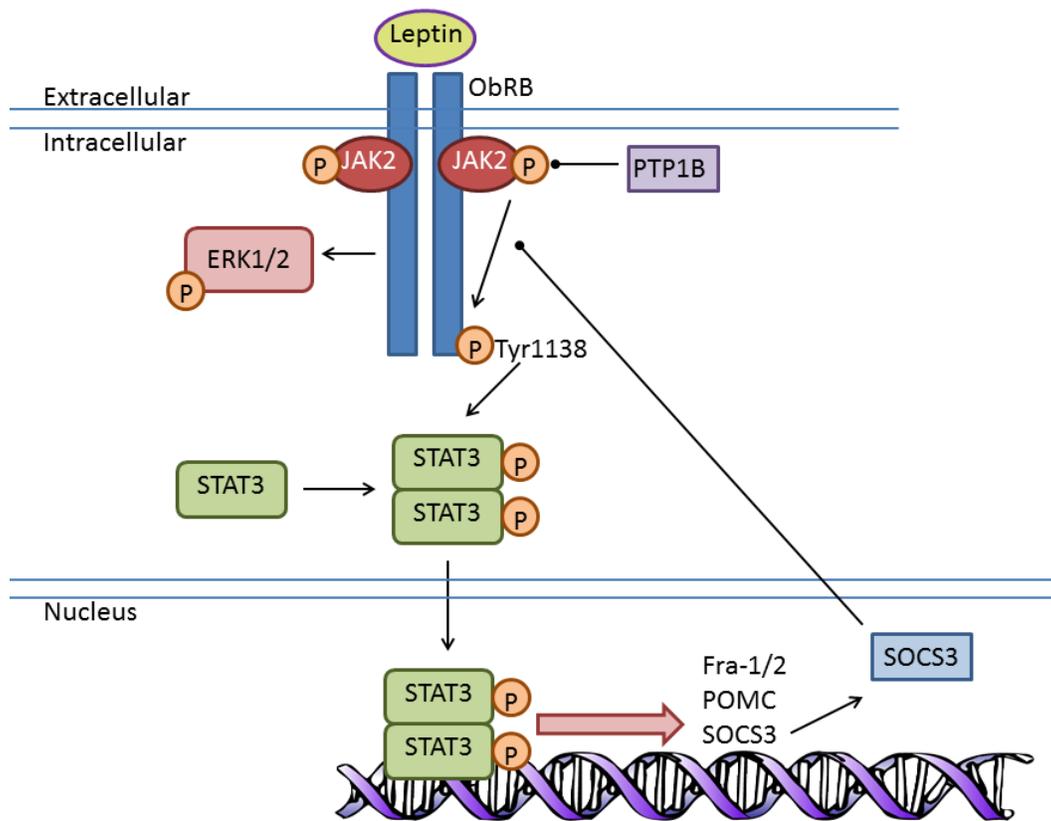
melanocortin 3/4 receptor (Schwartz et al., 2000), this peptide is able to reduce food intake. The *Pomc* gene is transcribed as a result of leptin, which can also drive the production of other anorexigenic proteins such as cocaine- and amphetamine-related transcript (CART) and corticotrophin releasing hormone (CRH; Arora and Anubhuti, 2006). Specific sets of neurons within ARC express POMC and CART together, are activated by leptin and induce satiety. Specific deletion of ObRB from these neurons causes hyperphagia and obesity (Dhillon et al., 2006; Balthasar et al., 2004). Leptin acting within the ARC also inhibits and reduces the production of orexigenic factors, including neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Elmquist et al., 1998; Baskin et al., 1999; Erickson et al., 1996).

In non-ARC regions of the brain, leptin acts through ObRB to mediate reductions in homeostatic food intake. Some of these regions include the ventromedial hypothalamus (Dhillon et al., 2006) and NTS (Hayes et al., 2009). Hedonic food intake has also been shown to be reduced by leptin through the dopaminergic mesolimbic system, namely the ventral tegmental area and accumbens nucleus (Leinninger et al., 2011; Hommel et al., 2006; Fulton et al., 2006).

1.4.4.2   Energy expenditure

Although leptin is primarily known for its effects on satiety and food intake, leptin is best described as a hormone that causes a shift to negative body energy

balance, as it is able to increase energy utilization. Leptin increases energy expenditure through three major mechanisms: 1) increased metabolism (Dieguez et al., 2011); 2) increased locomotion (Mendoza et al., 2011); and 3) increased thermogenesis (Scarpace et al., 1998).

1.4.5 Leptin and sympathetic nervous activity

Leptin acts to augment the sympathetic nervous activity in part to complete its negative feedback loop with energy balance. That is, release of leptin due to energy intake will cause the activation of the sympathetic nervous system, which will cause lipolysis in white adipose tissue, thermogenesis in brown adipose tissue and elevated metabolic activity within the liver and skeletal muscles. These physiological mechanisms will cause net energy expenditure.

Given that leptin acts in many homeostatic systems, the increase in sympathetic tone by leptin also regulates many physiological functions. Bone resorption, for example, has recently been shown to be regulated by the effect of leptin on the sympathetic nervous system (Eleftriou et al., 2005). Likewise, leptin is able to increase sympathetic nervous activity to the kidney and arterial vasculature to cause an increase in blood pressure (Mark et al., 2009). These effects may be caused by an inhibition of the baroreceptor reflex (Ciriello, 2013a, b; Arnold et al., 2009). Injections of leptin into the ARC increases sympathetic effects on the hindlimb and kidney, as well as the brown adipose tissue (Rahmouni and Morgan, 2007; Montaro et al., 2005). The role of leptin in ARC on

sympathetic outflow to both kidney and cardiovascular system appears to be dependent on the melanocortin system (do Camo et al., 2011; Morgan et al., 2008).

1.4.6 Leptin and neuroplasticity

Recent advances in the understanding of neuroplasticity in response to hormones such as leptin have provided a novel mediator of homeostatic functions such as food intake and sympathetic tone. Plastic alterations within specific brain regions such as ARC (McNay et al., 2013) and NTS (Kline, 2008) have demonstrated that these regions undergo dynamic regulation of function, neural morphology and synaptogenesis.

The role leptin may play in neuroplasticity and synaptic plasticity has only recently come to light. Leptin receptor has been localized to, not only somato-dendritic regions of neurons, but also axonal processes and synapses (Shanley et al., 2002). Animals deficient in leptin have electrophysiological deficits in long-term potentiation and long-term depression, both of which are important functional outcomes of neuroplasticity (Li et al., 2002). *In vivo,* leptin induces long-term potentiation (Wayner et al., 2004), which is dependent on N-methyl-D-aspartate (NMDA) receptor function (Collingridge et al., 1983). Leptin augments NMDA receptor signaling, increasing intracellular calcium influx (Shanley et al., 2001; Harvey et al., 2005). Likewise, there is a crucial role for GluN2 subunit of NMDA receptors in mediating the excitatory transmission of leptin (Moult and

Harvey, 2011). It appears that in adult life, the neuroplastic effect of leptin on synaptic strength is mediated by phosphoinositide-3 kinase, whereas at younger points of development this effect is likely due to ERK1/2 (Moult and Harvey, 2011).

In addition to the functional neuroplasticity associated with leptin, there are also significant morphological remodelling events that have been shown to occur, which likely contribute to the overall ability of leptin to modulate neuroplasticity (Maletic-Savatic et al., 1999). Leptin is able to increase the density and motility of dendrites, and alter the actin cytoskeleton (O'Malley et al., 2005, 2007; Ning et al., 2006). Both of these alterations are important for neurite outgrowth, as well as synaptogenesis (Fiala et al., 1998; Munno and Syed, 2003). Such alterations have been shown to be related to changes in growth-associated protein 43 (GAP-43) and the synaptic marker synaptophysin (Bottner et al., 2013; Routtenberg, 1985). These morphological alterations are mirrored in activity-dependent neuroplasticity (Fukazawa et al., 2003).

Neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) have recently been implicated in the satiety-inducing effects of leptin in both the hypothalamus and NTS (Liao et al., 2012; Spaeth et al., 2013). It appears these neuroplastic effectors exist downstream from the leptin receptor, and may be necessary for leptin to exert its effect (Liao et al., 2012).

1.4.7 Leptin resistance

When leptin was first discovered in 1994, it was believed the satiety-inducing hormone would act to resolve positive body energy balance syndromes like obesity. However, the finding of hyperleptinemia, as opposed to hypoleptinemia, in obese subjects suggested otherwise. An increase in hormone concentration with a reduction in its effect is suggestive of a resistance to that hormone, as seen with insulin in type II diabetes mellitus (Friedman, 2010). Application of leptin to obese mice have varying sensitivities to the hormone, with the leptin-deficient *ob/ob* mouse being the most sensitive and the leptin-receptor mutated *db/db* being the least sensitive (Halaas et al, 1997). This effect is mirrored in humans, where obese leptin-deficient people are sensitive to leptin, while most other forms of obesity are leptin-resistant. Primary leptin-deficiency in human populations is extremely rare (Farooqi et al., 1999), but secondary leptin-deficiency is slightly more common, arising from lipodystrophy and hypothalamic amenorrhea. In all cases, administration of recombinant leptin was able to resolve many resulting neuroendocrine, reproductive and metabolic issues (Farooqi et al., 1999; Oral et al., 2002; Welt et al., 2004).

The mechanisms associated with leptin resistance are not completely understood. As is found with insulin resistance, many changes in leptin signaling and transport systems may accumulate to mediate the resistant state. Hyperleptinemia associated with leptin resistance may play an important role as both a cause and result of the pathophysiology, given that overexpression of leptin within the brain induces leptin resistance, and blockade of leptin receptors

in obese animals exaggerates diet-induced obesity (Scarpace and Zhang, 2007). The resistance to leptin may be the result of decreased transport across the blood-brain barrier (Katsin et al., 1999; Banks et al., 2002; Burguera et al., 2000; Dube et al., 2000), and obese humans have a reduced cerebrospinal fluid-serum ratio of leptin despite concomitant hyperleptinemia (Caro et al., 1996; Schwartz et al., 1996).

A reduction in downstream signaling of leptin within ARC may also play an important role in leptin resistance, either at the level of the receptor availability, reduced post-receptor activities, or by augmented negative feedback at the receptor (Friedman et al., 2010; Mark, 2013).

Despite this reduced leptin effect on satiety, thus causing positive energy balance, it has been noted that the sympathetic effects of leptin are maintained during leptin resistance (Rahmouni et al., 2002; Correira et al., 2002). This has given rise to the concept of a selective leptin resistance in obesity, and has recently garnered attention as a contributor to obesity-related hypertension (Kuo et al., 2001). The selective nature of leptin resistance is poorly understood, but may be the result of alternative signaling mechanisms in sympathetic-related neurons and brain regions following leptin binding (Mark, 2013).

1.5 HYPOTHESIS AND OBJECTIVES

1.5.1 Hypothesis

We hypothesize that IH in rats will lead to metabolic and hypertensive cardiovascular phenotypes, which will be associated with alterations in brain regions known to regulate body energy balance and the cardiovascular system. This thesis will focus largely on manipulations of various hormones released in response to IH, and how they may mediate or affect the phenotypes observed.

1.5.2 Objectives

The major objectives of this thesis are as follows:

1) characterize the body energy balance phenotype that may be associated with both short- and long-term IH and its concomitant alterations in hormonal release;

2) determine alterations in the ARC of animals exposed to short- and long-term IH that may be associated with body energy balance and the role leptin signaling may play in mediating these effects;

3) measure cardiovascular alterations, including baroreceptor reflex function, following short- and long-term IH, which may be associated with

alterations in neuroplasticity within NTS, and the role leptin may play in the short-term changes, and

4) determine the role of angiotensin II in modulating leptin release and signaling in the carotid body following IH.

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CHAPTER 2

EFFECTS OF ACUTE INTERMITTENT HYPOXIA ON ENERGY

BALANCE AND HYPOTHALAMIC FEEDING PATHWAYS

2. CHAPTER 2

2.1 CHAPTER SUMMARY

This study was done to investigate the effects of acute intermittent hypoxia (IH) on metabolic factors associated with energy balance and body weight, and on hypothalamic satiety-inducing pathways. Adult male Sprague-Dawley rats were exposed to either 8h IH or normoxic control conditions. Food intake, locomotion and body weights were examined after IH. Additionally, plasma levels of leptin, adiponectin corticosterone, insulin and blood glucose were measured following exposure to IH. Furthermore, adipose tissue was removed and analyzed for leptin and adiponectin content. Finally, the hypothalamic arcuate nucleus (ARC) was assessed for alterations in protein signaling associated with satiety. IH reduced body weight, food intake and active cycle locomotion without altering adipose tissue mass. Leptin protein content was reduced while adiponectin content was elevated in adipose tissue after IH. Plasma concentration of leptin was significantly increased while adiponectin decreased after IH. No changes were found in plasma corticosterone, insulin and blood glucose. In ARC, phosphorylation of signal transducer and activator of transcription-3 and pro-opiomelanocortin (POMC) expression were elevated. In addition, POMC-expressing neurons were activated as determined by immediate early gene FRA-1/2 expression. Finally, ERK1/2 and its phosphorylation were reduced in response to IH. These data suggest that IH induces significant alterations to body energy balance through changes in the secretion of leptin which exert effects on satiety-inducing pathways within the hypothalamus.

2.2 INTRODUCTION

While white adipose tissue is the major storage site for triglycerides in the body, this organ also appears to play a role in endocrine function, partly through the production and release of a class of hormones known as adipokines. Adipokines are used by the body to provide information regarding energy balance, which in turn may influence thermogenic activity, food intake and glucose homeostasis (Ahima and Osel, 2008). Leptin, a 16 kDa hormone produced by the *ob* gene (Halaas et al., 1995), is an adipokine that functions as a satiety-inducing hormone. The primary site of secretion of leptin into the bloodstream is the white adipose tissue, and this secretion generally occurs directly in relationship to the white adipose tissue mass (Maffei et al., 1995). However, the release of leptin into the circulation can be altered by a number of different stimuli, including hypoxia (Reinke et al., 2011; Sherry et al., 2009) and acute intermittent hypoxia (IH) (Messenger et al., 2012). Hypoxia elevates leptin production and release from white adipose tissue in both *in vitro* (Grosfield et al., 2002) and *in vivo* (Sherry et al., 2009). Similarly, acute IH has been shown to increase the circulating levels of leptin (Messenger et al., 2012). Although the functional role of this upregulation of the leptinergic system is unknown, it may be involved in the regulation of the sympathetic nervous system activity (Mark et al., 2009; Ciriello and Moreau, 2012), and this may be mediated partly through its effects on hypothalamic neurons (Harlan et al., 2011). Leptin not only reduces food intake largely by acting on pro-opiomelanocortin (POMC) neurons of the arcuate nucleus (ARC; Hill et al., 2008), but these POMC neurons may also

affect blood glucose levels (Berglund et al., 2012; Shi et al., 2008). Leptin binds to POMC neurons through the long-form leptin receptor (Ob-Rb), resulting in the homodimerization and phosphorylation of signal transducer and activator of transcription-3 (STAT3; Calvino et al., 2012). Phosphorylated STAT3 (pSTAT3) translocates to the nucleus of these neurons and alters gene transcription of various targets including *Pomc* and immediate early genes (Bousquet et al., 2000).

As adipose tissue is responsive to hypoxia, and that many factors produced by adipose tissue are related to energy balance, this study was done to determine whether acute 8 h IH altered the signaling of the adipose, resulting in alterations in energy balance.

2.3 METHODS AND MATERIAL

2.3.1 Animals

Adult, male Sprague-Dawley rats (320 – 400 g: n = 48) were purchased from Charles River Canada and housed singly at a temperature of 22°C and 60% relative humidity with access to food and water available *ad libitum*, except during the acute 8 h IH or normoxic (Norm) exposure, in 12h light/dark cycle conditions. Animals were handled in accordance with the guidelines set forth by the Canadian Council on Animal Care and the Animal Use Committee at the University of Western Ontario.

2.3.2 Groups

Animals were divided into different subsets: the first subset (n=7 per condition) was used to determine physiological measures including food and water intake and body weight changes; the second subset (n=7 per condition) was used for active cycle locomotion studies; the third subset (n=7 per condition) was used for immediate sacrifice following exposure, from which plasma samples (used for hormone analysis), blood glucose, adipose tissue and brains were removed; the final subset of animals (n=3 per condition) were perfused immediately following exposure, and were used for immunohistochemistry and immunofluorescence.

2.3.3 IH or Norm exposure

Animals were exposed to 8 h (0900-1700) of acute IH or Norm during their sleep cycle, as previously described (Messenger et al., 2012). In brief, animals were placed in a chamber (35 L volume) consisting of four separate animal tubes (10 cm diameter by 35 cm length) and a zero-pressure escape valve. For IH-exposed animals, a computer that regulated solenoid valves altered the input of N_2 or room air to generate IH and Norm conditions. The gases were pushed through the system using fans and passed through a mixing chamber prior to entering the animal tubes. Flows of N_2 and room air were set to be equal, requiring the same amount of time to drop from 21% to 6.5% O_2, as required returning to 21% from 6.5% O_2. Animals were exposed to 80s hypoxia (6.5% O_2) followed by 120s normoxia. The levels of O_2 and CO_2 were monitored by sensors on the chamber, which relayed information back to the computer to ensure proper cycling. Conditions within the chamber were isobaric (770 ± 11 mmHg) and eucapnic (<0.1% CO_2). Norm animals were exposed to identical chambers with only room air input during cycling. No alterations in sleep duration or locomotion were observed between IH or Norm animals during exposure.

2.3.4 Measures of body weight, food intake and water intake

Animals were weighed immediately before and after IH or Norm exposure. These values were used to calculate body weight changes during the IH or Norm exposure period and 24 h body weight change. Additionally, food and water

intake measurements were recorded for the 16 h period immediately following exposure

2.3.5 Locomotion assay

In a subset (n=7 per group), immediately following acute IH or Norm exposure, animals were placed in home cages with *ad libitum* access to food and water. Thirty minutes into the dark (active) cycle (1930h), animals were placed into large cages (60 cm x 40 cm) with a floor 4x5 grid system in the dark (red light on). Animals were acclimatized for 10 min to the testing cage. Over a five-minute period, the number of crosses of a line by an animal was determined by two independent, blinded observers to determine horizontal locomotion. At the same time, vertical locomotion was determined by the number of rearing events. An average value was then calculated from these two observers for both horizontal and vertical locomotion. These animals were not used for determination of food or water intake.

2.3.6 Blood glucose measurement

Immediately following exposure conscious animals had blood glucose measured three times using an Accu-Check Aviva glucometer (Roche Diagnostics Canada; Laval, QC) from a tail vein puncture.

2.3.7 Plasma collection and enzyme immunoassays

Subgroups of rats exposed to IH (n=7) or Norm (n=7) were immediately sacrificed under equithesin anaesthesia [0.3 ml/100 g b.w.; i.p. (2.88 mg/100 g b.w. sodium pentobarbital; 12.79 mg/100 g b.w. chloral hydrate; 6.37 mg/100 g b.w. MgSO4; 0.12 ml/100 g b.w. propylene glycol; 0.03 ml/100 g b.w. ethanol)] immediately following exposure. Blood samples were collected by cardiac puncture in the presence of 7% ethylenediaminetetraacetic acid at a volume of 10 µl/ml blood. This blood was immediately centrifuged at 10 000 RPM for 10 min at 4 °C to isolate the aqueous plasma. This aqueous plasma phase was removed and stored frozen at -80 °C until analyzed for hormone content. Plasma samples were analyzed using enzyme immunoassays for rat leptin (sensitivity: 67.2 pg/ml; Enzo Life Sciences; Farmingdale, NY), adiponectin (sensitivity: 0.12 ng/ml; Phoenix Pharmaceuticals; Burlingame, CA), corticosterone (sensitivity: 26.99 pg/ml; Enzo Life Sciences; Farmingdale, NY) and insulin (sensitivity: 0.12 ng/ml; Alpco Diagnostics; Salem, NH), according to manufacturer instructions. Enzyme immunoassay plates were read on a SpectraMax M5 plate reader using SoftMax Pro v.5 microplate analysis software (Molecular Devices; Sunnyvale, CA).

2.3.8 Tissue collection and preparation

Immediately after exposure to IH (n=7) or Norm (n=7), additional subgroups of the animals were sacrificed under equithesin anaesthesia and the brain and retroperitoneal fat pad were removed and frozen at -80 °C until analyzed. Brains

were sectioned at 100 µm in a cryostat (Bright Instruments; Cambridgeshire, UK) until ARC was identified using cytoarchitectural landmarks and a rat brain atlas (Paxinos and Watson, 1986). Using a circular 1 mm (internal diameter) micropunch tool, bilateral punches of ARC were made to a depth of 0.5 mm and the frozen tissue was then homogenized in cold radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate) with protease inhibitor cocktail (Roche Applied Science; Laval, QC) by an electric homogenizer (VWR International; Radnor, PA). Homogenates were then sonicated over three passages for 15s each on ice (55%; Sonic Dimembrator Model 150; Fisher Scientific). Samples were then rotated for 10 min at 4 °C and centrifuged at 4°C for 20 min at 14 000 RPM. Adipose tissue samples were taken while frozen and processed using the same procedure. Protein content of homogenates was quantified using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories; Hercules, CA). Protein samples were added to 25% LDS sample buffer and 10% reducing buffer (Life Technologies; Burlington, ON) and water to a standard protein concentration of 1.67 mg/ml.

2.3.9 Western blots

Electrophoresis was carried out using a 10% discontinuous polyacrylamide Bis-Tris gel (Life Technologies; Burlington, ON), followed by standard protein immunoblotting techniques. For each animal, 25 µg of protein of each sample

was loaded. Electrophoresis was carried out at 200 V and terminated when the dye front reached the bottom of the gel. Proteins were transferred to a polyvinylidene fluoride membrane using a wet transfer method in the presence of methanol and SDS (50 mM Tris, 40 mM glycine, 0.3% SDS, 20% methanol) and wet transfer apparatus (Mini Trans-Blot Electrophoretic Transfer Cell; Bio- Rad Laboratories; Hercules, CA) at 100 V for 2 h. After transfer, the membrane was washed in Tris-buffered saline + Tween-20 (TBST; 20 mM Tris, 0.5 M NaCl, 0.1% Tween-20; pH 8.0) blocked for 1 h with 5% skim milk made in TBST buffer at room temperature. The membrane was then incubated with primary antibodies diluted in skim milk over night at 4 °C. The following day, the membrane was washed with TBST before being incubated with horseradish peroxidase-conjugated secondary antibodies-specific to the appropriate host of the primary antibody being analyzed, for 1 h at room temperature. For detection, the membrane was washed with TBST, followed by distilled water and then detected using a horseradish peroxidase substrate ECL chemiluminescence system (Luminata Forte, EMD Millipore; Billerica, MA). Blots were visualized using a VersaDoc imaging system (Bio-Rad Laboratories; Hercules, CA) and analyzed using ImageLab v.3.0 (Bio-Rad Laboratories; Hercules, CA). All comparisons presented were made within the same blot.

2.3.10 Immunohistochemistry

Immediately following acute IH (n=3) or Norm (n=3) exposure, rats were deeply anesthetized with equithesin and perfused transcardially with 200-300 ml 0.9% physiological saline, followed by 450 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Brains were removed and placed overnight in fixative at 4ºC. Brains were then gradually dehydrated through a series of alcohols, and placed in xylene followed by paraffin wax. Serial, transverse sections (6 µm) through the region of ARC were cut on a microtome, mounted on double-gelatinized microscope slides and placed on a slide warmer. Tissue sections were later de-paraffinized in xylene and rehydrated using graded alcohol solutions. Sections were equilibrated using three 20 min washes of PBS (pH 7.4), and then underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate, 0.05% Tween; pH 6.0) heated to 90-95ºC in a microwave for 15 min (von Boguslawsky, 1994). Slides were rinsed and endogenous peroxidase activity was inhibited by exposing the sections to a 1% hydrogen peroxide solution for 10 min. Sections were washed in PBS and allowed to incubate overnight at 22ºC in primary polyclonal rabbit anti-rat POMC antibody in PBS/0.3% Triton-X 100 and 4% normal goat serum. The sections were rinsed in PBS and exposed to goat anti-rabbit IgG for 1 h, followed by 75 min in ABC Elite Kit reagent (Vector Laboratories; Burlingame, CA). Following three rinses in PBS, the sections were immersed for 5 min in a solution of 0.05% 3,3'-diaminobenzidine containing 0.01% hydrogen peroxide, resulting in a red-brownish reaction product. The sections then underwent a similar protocol, but

using an overnight incubation in primary polyclonal rabbit anti-rat FRA-1/2 antibody, after an avidin-biotin block (Vector Laboratories; Burlingame, CA). FRA-1/2 was detected using a solution of 0.05% 3,3'-diaminobenzidine containing 0.015% hydrogen peroxide and 0.05% nickel ammonium sulphate in PBS, resulting in a dense, black reaction product. Sections were rinsed and coverslipped. Some adjacent tissue sections were rehydrated, stained with thionin and cover-slipped for identification of cytoarchitectonic boundaries. Controls for immunoreactivity included forebrain brainstem sections processed following the omission of the primary antibody or preadsorption of the primary antibody with the appropriate antigen (Messenger et al., 2012). Under these conditions no POMC or FRA-1/2 immunoreactivity was demonstrated.

Cells containing POMC- and FRA-1/2-like immunoreactivity were identified on sections through the region of ARC using bright-field microscopy (Letiz Diaplan), and digital images were obtained with a Nikon DS-Fil camera and NIS Elements Basic Research 3.0 software (Nikon Canada, Mississauga, ON, Canada). Cell counts of POMC-containing neurons co-expressing FRA-1/2-like immunoreactivity were determined by two independent, blinded observers and averaged to calculate a final value for each animal.

2.3.11 Immunofluorescence

Immunofluorescence was performed in a similar manner as described above for immunohistochemistry, except paraffin-embedded sections through the region of

ARC were exposed to specific antibodies for POMC and FRA-1/2 overnight, exposed to either biotinylated secondary antibody, and then exposed to streptavidin-linked Texas Red (1:100; GE Healthcare; Buckinghamshire, UK) or Alexa-488-linked secondary antibodies.

Cells containing POMC- and FRA-1/2-like immunofluorescence were identified throughout the region of ARC using a microscope fitted with various filters to detect Texas Red and Alexa-488. Digital images were obtained with a Nikon DS-Fi1 camera and NIS Elements Basic Research 3.0 Software (Messenger et al., 2012).

2.3.12 Antibodies

For western blots the following antibodies were used: rabbit anti-β-actin-HRP (1:50 000; A3854, Sigma-Aldrich; St. Louis MO), rabbit anti-GAPDH (1:2500; sc-25778, Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-leptin (1:1000; sc-843, Santa Cruz Biotechnology; Santa Cruz, CA), chicken anti-ObRB (1:10 000; CH14104, Neuromics; Edina, MN), rabbit anti-FRA-1/2 (1:2000; sc-605, Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-STAT3 (1:2000; #9132, Cell Signaling; Boston, MA), rabbit anti-pSTAT3 (Tyr705) (1:1000; #9131, Cell Signaling; Boston, MA), rabbit anti-ERK1/2 (1:2000; #9102, Cell Signaling; Boston, MA), rabbit anti-pERK1/2 (Thr 202/Tyr204) (1:1000; #9101, Cell Signaling; Boston, MA), rabbit anti-adiponectin (1:5000; AB3267P, Millipore; Billerica, MA), rabbit anti-POMC (1:2000; RayBiotech; Norcross GA), donkey

anti-rabbit IgG-HRP (1:10 000; 711-035-152, Jackson Immunoresearch; West Grove, PA), donkey anti-chicken IgY-HRP (1:10 000; 703-035-155, Jackson Immunoresearch; West Grove, PA). For immunohistochemistry and immunofluorescence the following antibodies were used: rabbit anti-POMC (1:10 000; H-029-030, Phoenix Pharmaceuticals; Burlingame, CA), rabbit anti-FRA-1/2 (1:1000; sc-253, Santa Cruz Biotechnology; Santa Cruz, CA), goat anti-rabbit IgG-biotin (1:500; BA-1000, Vector Laboratories; Burlingame, CA) goat anti-rabbit IgG-Alexa-488 (1:200; 711-545-152, Jackson Immunoresearch; West Grove, PA).

2.3.13 Statistics and analysis

Differences between acute IH and Norm groups were determined by unpaired, two-tailed Student t-test, and a p-value < 0.05 was taken to indicate statistical significance. All values are expressed as mean ± standard error. All bar charts were made using GraphPad Prism v.5 graphing software (GraphPad Software; La Jolla, CA).

2.4 RESULTS

2.4.1 Body weight, adipose tissue mass, food and fluid intake and locomotion after acute IH

Animals in acute IH and Norm groups did not differ in starting body weight (365 ± 9 g and 366 ± 10 g, respectively). Animals immediately after exposure to acute 8 h IH had significantly ($p < 0.01$) greater body weight loss (-28 ± 2 g) compared to those exposed to Norm conditions (-13 ± 1 g). However, these differences in body weight change 24 h after the initiation of the exposure were reversed as animals exposed to acute IH (-5 ± 3 g) were not different from animals that were exposed to Norm conditions (1 ± 3 g). No changes were found in retroperitoneal (IH, 4.6 ± 1.2 g; Norm, 5.0 ± 1.1 g) or epididymal (IH, 2.8 ± 0.7 g; Norm, 3.2 ± 0.9 g) adipose tissue mass (Fig 2.1).

Despite the overnight body weight regain, food intake was significantly lower ($p < 0.01$) in acute IH animals (total food intake, 21.1 ± 2.1 g/16 h; food intake normalized to body weight, 5.9 ± 0.5 g/100 g body weight/16 h) compared to animals exposed to Norm conditions (total food intake, 35.4 ± 5.5 g/16 h; food intake normalized to body weight, 9.4 ± 1.3 g/100 g body weight/16 h). Water intake was not altered in response to acute IH exposure (total water intake, 42.6 ± 3.5 ml/16 h; water intake normalized to body weight, 12.0 ± 0.8 ml/100 g body weight/16 h) compared to Norm conditions (total water intake, 39.5 ± 7.0 ml/16 h; water intake normalized to body weight, 10.5 ± 1.9 ml/100 g body weight/16h).

Figure 2.1 Changes in body weight and fat mass following IH.

Acute IH causes a greater reduction in body weight compared to Norm conditions, independent of alterations in adipose tissue mass. (a), line graph indicating significant ($p<0.01$) body weight loss as a result of the IH compared to Norm. This body weight loss is normalized 24h after the initiation of IH, and is not different from Norm. Bar graphs showing (b) retroperitoneal and (c) epididymal adipose tissue mass were not different following IH exposure compared to Norm animals. Data shown are means ± SEM. *, $p<0.05$. n = 7-11 per group.

a

Time Following Initiation of Exposure (h)

b

c
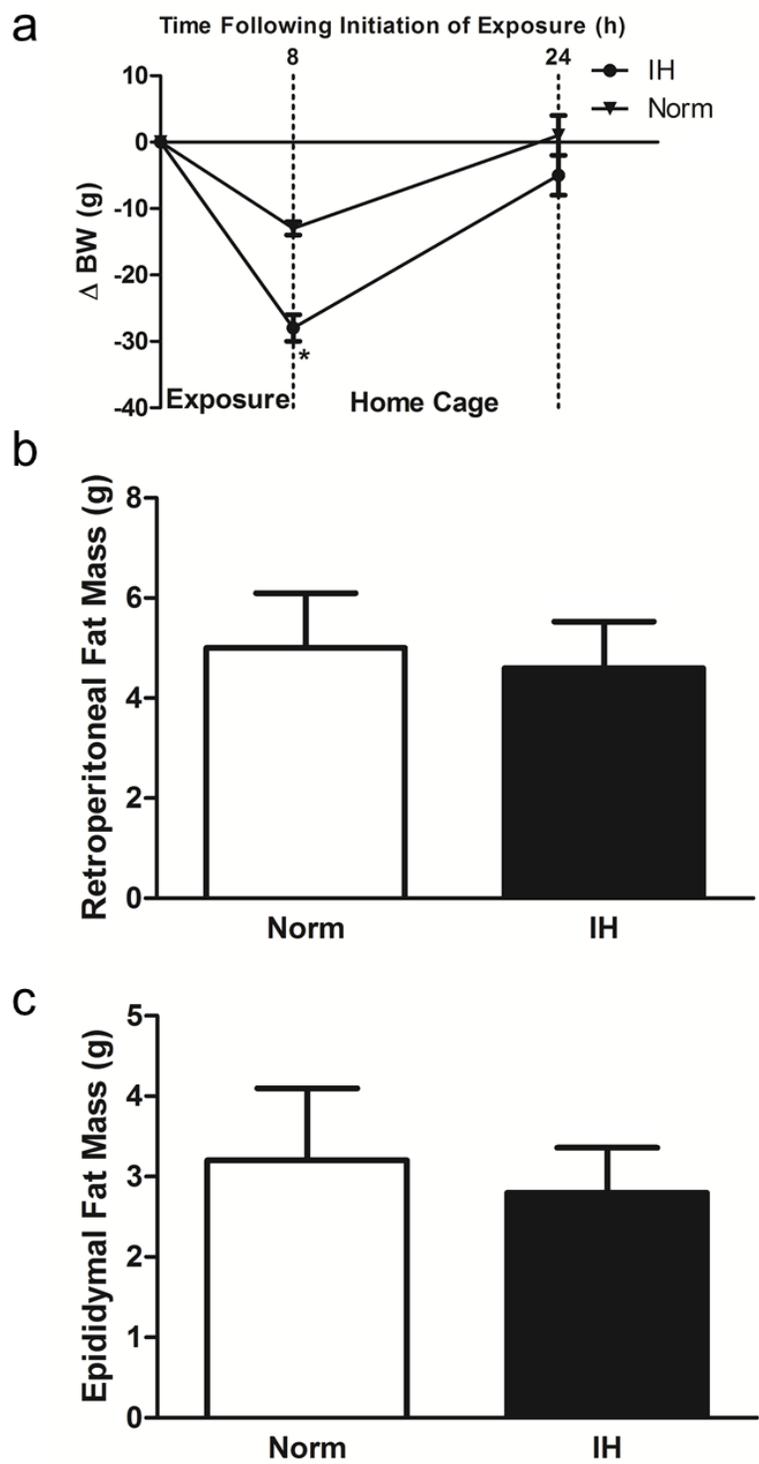
Animals exposed to acute IH had significantly ($p < 0.05$) reduced horizontal locomotion (IH, 88.5 ± 4.1 line crossings/5 min; Norm, 115 ± 9.7 line crossings/5 min). In contrast, vertical locomotion (IH, 28.0 ± 2.6 rearings/5 min) compared to Norm (32 ± 3.4 rearings/5 min) were not different. These data are summarized in Figure 2.2.

2.4.2 Plasma leptin, adiponectin and leptin:adiponectin ratio are altered by acute IH

Plasma leptin concentrations in animals exposed to acute IH (10.0 ± 1.3 ng/ml) were significantly ($p < 0.05$) greater than in animals exposed to Norm conditions (3.2 ± 1.3 ng/ml) immediately following exposure. Plasma adiponectin concentration was significantly ($p < 0.05$) less in acute IH compared to Norm exposure (2.2 ± 0.1 µg/ml and 2.6 ± 0.1 µg/ml, respectively). The ratio of the concentration of leptin to adiponectin in the plasma of the IH group was significantly ($p < 0.01$) higher than in the Norm group (0.0045 ± 0.0008 and 0.0012 ± 0.0003, respectively). Plasma corticosterone concentrations were not changed by acute IH compared to Norm control (4.8 ± 1.2 ng/ml and 5.3 ± 1.1 ng/ml, respectively). Similarly, plasma insulin concentrations were unchanged in the acute IH group compared to Norm conditions (6.4 ± 0.8 ng/ml and 7.2 ± 0.6 ng/ml, respectively). Furthermore, blood glucose levels immediately following acute IH or Norm exposure were not different from each other (8.2 ± 1.0 mmol/L and 7.9 ± 1.3 mmol/L, respectively). These data are summarized in Figure 2.3.

Figure 2.2 Changes in food intake and locomotion following IH.

Acute IH results in reduced food intake and locomotion compared to Norm conditions. Bar graphs indicating overnight food intake (a), standard food intake (b), water intake (c), standard water intake (d), horizontal locomotion measure (e), and vertical locomotion measure (f) between IH and Norm animals. Data shown are means ± SEM. *, p<0.05. n = 11 per group.
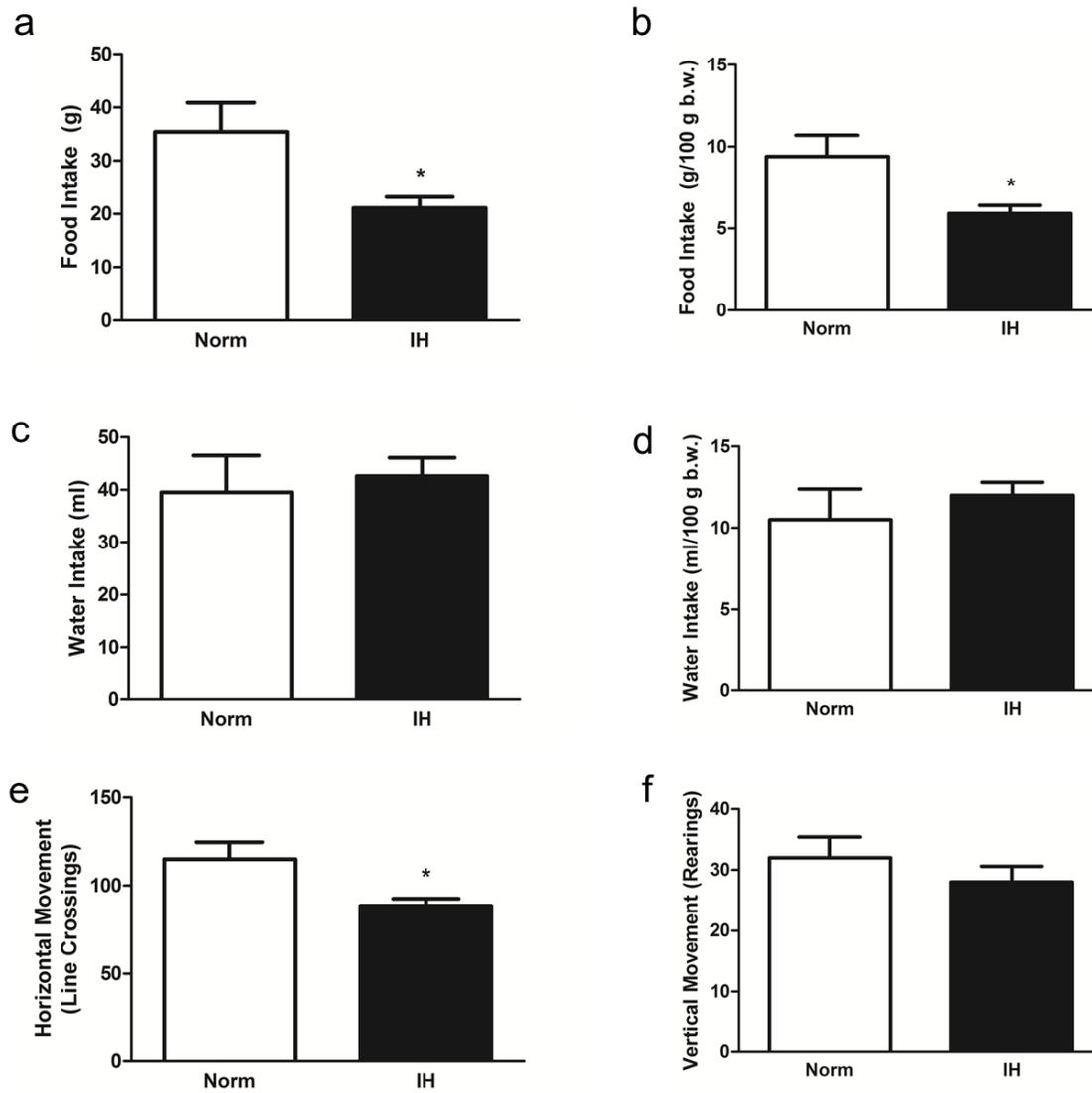
Figure 2.3 Changes in plasma hormones in response to IH.

Plasma hormones are altered after acute IH. Bar graphs showing plasma levels of leptin (a), adiponectin (b), leptin:adiponectin ratio of plasma concentrations (c), corticosterone (d), insulin (e), and blood glucose (f) immediately after IH or Norm exposure. Data shown are means ± SEM. *, $p < 0.05$. n = 7 per group.
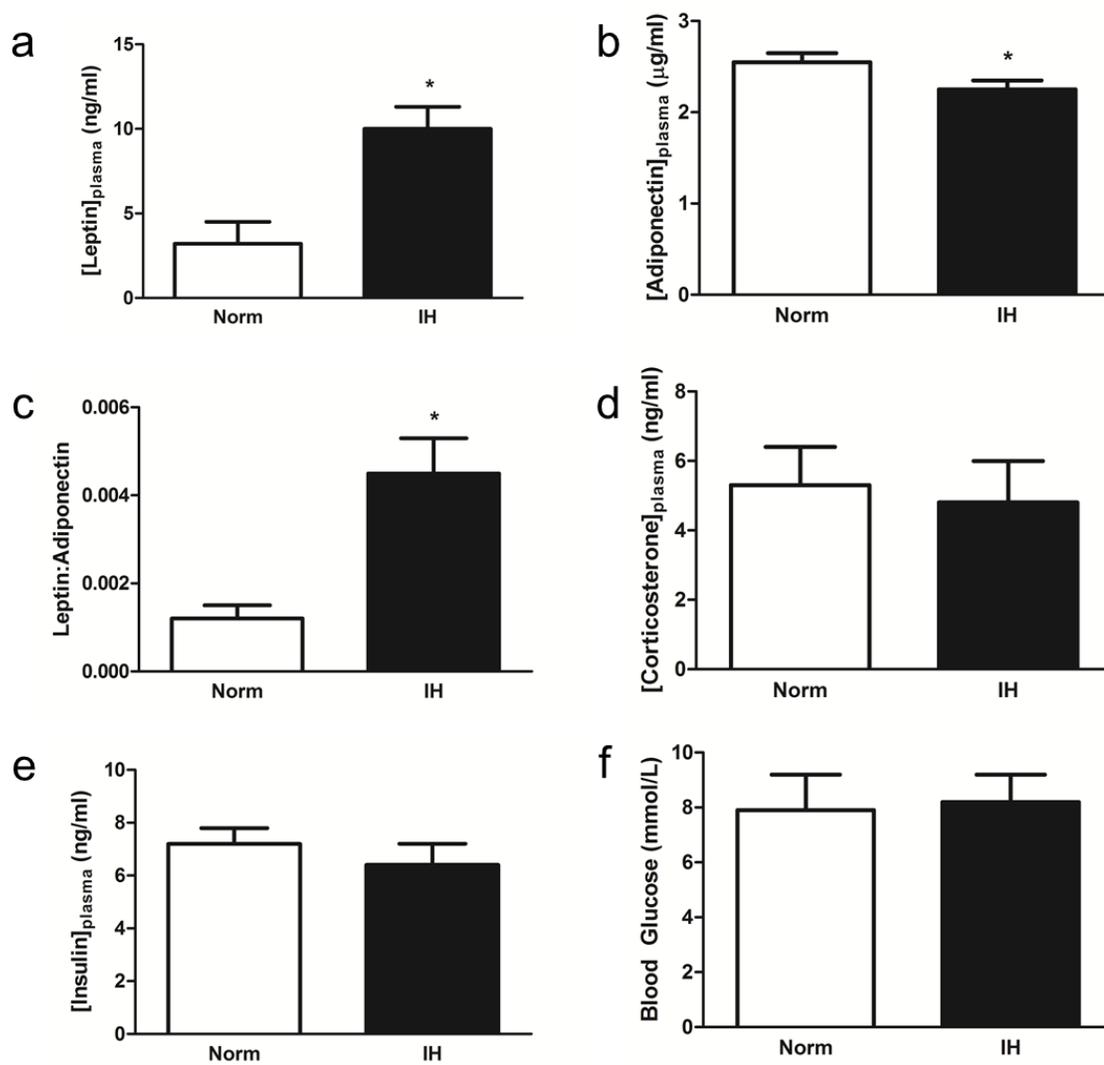
2.4.3 Adipose tissue content of leptin and adiponectin protein is altered as a result of acute IH exposure

As summarized in Figure 2.4, retroperitoneal adipose tissue displayed reduced (p < 0.01) expression levels of leptin protein after acute IH exposure compared to Norm animals (0.01 ± 0.01 and 0.28 ± 0.05, respectively). On the other hand, the protein expression of adiponectin in this tissue was significantly ($p < 0.05$) elevated between acute IH and Norm conditions (0.11 ± 0.02 and 0.05 ± 0.01, respectively).

2.4.4 IH exposure alters signals associated with leptin signaling within ARC

Within ARC, acute IH exposure increased the expression of phosphorylation of STAT3 protein approximately 8-fold ($p < 0.001$) over Norm (0.86 ± 0.05 and 0.11 ± 0.05, respectively) without changing total STAT3 protein expression (0.32 ± 0.06 and 0.34 ± 0.05, respectively). A significant ($p < 0.05$) decrease in the total amount of ERK1/2 protein expression was also detected in ARC of acute IH exposed animals compared to Norm control (0.41 ± 0.08 and 0.56 ± 0.02, respectively). In addition, the expression level of phosphorylation of ERK1/2 protein was reduced between IH and Norm conditions when compared to total ERK1/2 (1.05 ± 0.09 and 1.41 ± 0.13, respectively) and compared to β-actin (0.42 ± 0.07 and 0.79 ± 0.08, respectively). On the other hand, acute IH did not alter SOCS3 protein expression in ARC, compared to Norm (0.45 ± 0.07 and 0.38 ± 0.08). Additionally, acute IH did not induce an alteration in the expression

Figure 2.4 Adipose adipokine protein content following IH.

Adipose tissue protein content of leptin and adiponectin are altered by acute IH exposure. Bar graphs and representative blots indicating a reduction in retroperitoneal adipose tissue protein content of leptin (a) and an increase in adiponectin (b) protein content relative to GAPDH protein loading control, after IH compared to Norm. Data shown are means ± SEM. *, $p < 0.05$. n = 7 per group.

a

Leptin

GAPDH

Pixel Intensity (Leptin/GAPDH)

0.4

0.3

0.2

0.1

0.0

Norm   IH

*

b

Adiponectin

GAPDH

Pixel Intensity (Adiponectin/GAPDH)

0.15

0.10

0.05

0.00

Norm   IH

*
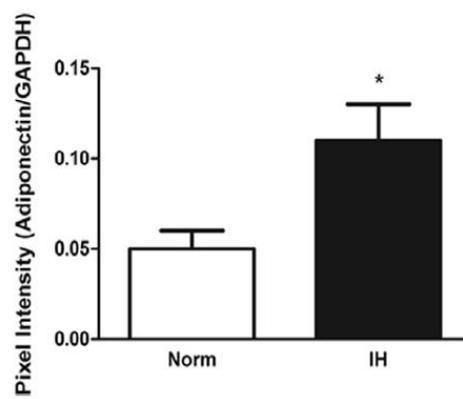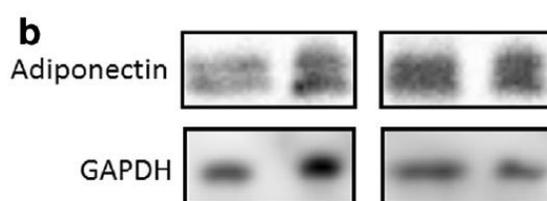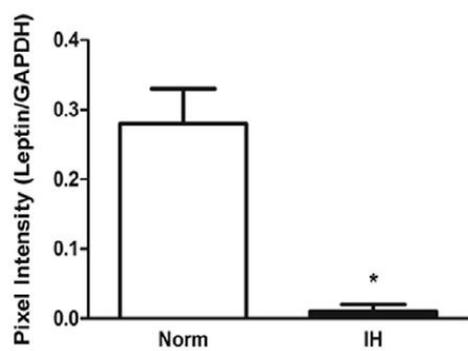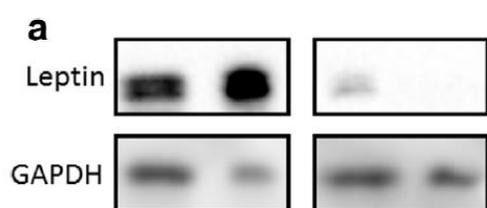
of the Ob-Rb protein compared to Norm control (0.32 ± 0.03 and 0.41 ± 0.05, respectively). These data are summarized in Figure 2.5.

2.4.5 Acute IH activates POMC-containing neurons and increases POMC protein within ARC

Exposure of animals to acute IH increased the number of POMC-like immunoreactive neurons that also expressed the immediate early gene product FRA-1/2 compared to Norm animals (15.1 ± 2.2% and 2.3 ± 2.5%, respectively) within ARC (Fig. 2.6). The expression of POMC protein within ARC was significantly ($p < 0.01$) elevated in animals exposed to IH compared to Norm (0.21 ± 0.07 and 0.08 ± 0.02, respectively; Fig. 2.6).

Figure 2.5 ObR related intracellular pathways activated following IH

Acute IH activates specific long-form leptin receptor (Ob-Rb)-related intracellular pathways in ARC. Bar charts and representative blots indicating relative protein content of phosphorylated STAT3 (a), phosphorylated ERK1/2 (b), SOCS3 (c) and Ob-Rb (d) within ARC of animals exposed to IH or Norm conditions. These values are expressed as a ratio to total β-actin protein. Data shown are means ± SEM. *, $p<0.05$. n=7 per group.

a

pSTAT3

STAT3

b

pERK1/2

ERK1/2

c

SOCS3

β-Actin

d

Ob-Rb

β-Actin
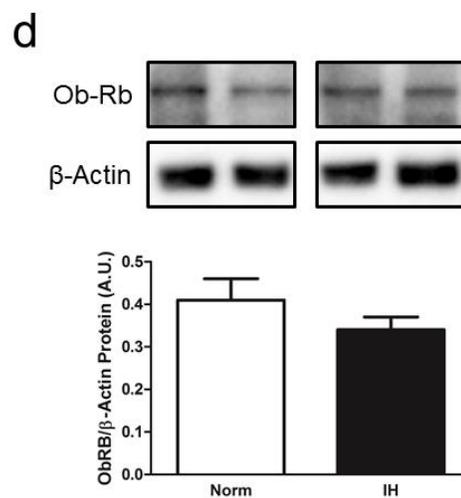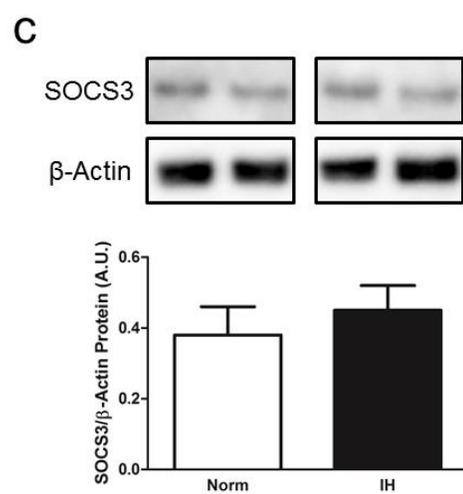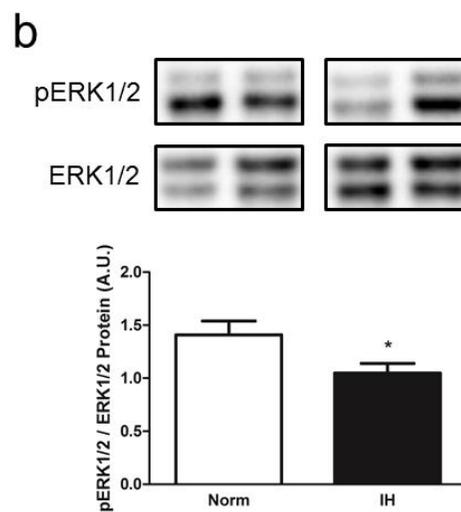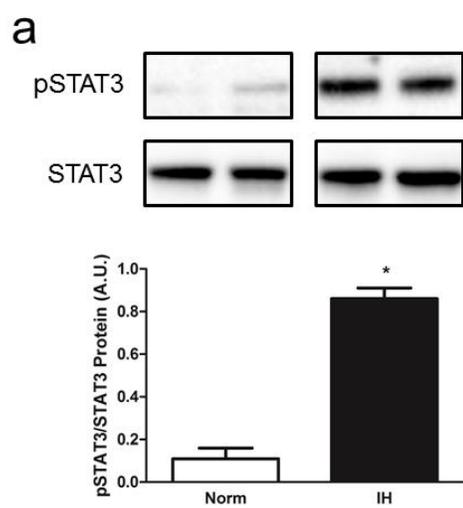
Figure 2.6 ARC POMC-containing cell activation following IH.

Acute IH increases activation of POMC-containing cells and POMC protein content in ARC. Photomicrographs of ARC indicating POMC-containing cells (brown), which display FRA-1/2 (black), a marker of neuronal activation, in both IH (a-b) and Norm conditions (c). Calibration marks represents 50 μm. (b) High-magnification image of inset in (a). (d) Immunofluorescent image of ARC following exposure to IH. Note that POMC immunoreactivity is shown with green fluorescent marker and FRA 1/2 immunoreactivity with Texas red fluorescent marker. In both (b) and (d) large open arrows indicate cells that contain both POMC- and FRA-1/2-like immunoreactivities, while small open arrows show example of Fra-1/2 only labelled cells and the small arrow shows examples of POMC only labelled cells. (e), shows a bar chart summary of the proportion of ARC POMC-containing cells that also express FRA-1/2-like immunoreactivity. (f), bar chart and representative blots indicating the relative protein content of POMC within ARC in IH and Norm animals. These values are expressed as a ratio to total β-actin protein. Data shown are means ± SEM. *, $p < 0.05$. n = 3 per group for immunohistochemistry; n = 7 per group for western blot analysis.
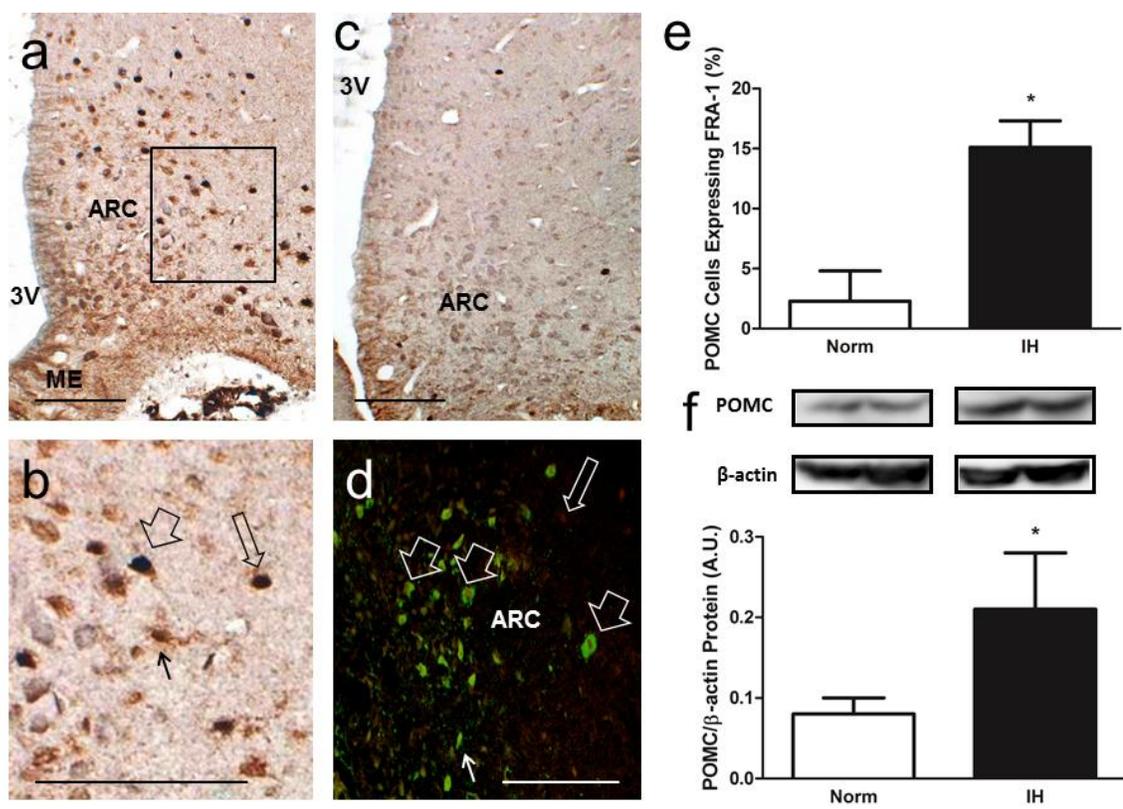
2.5 DISCUSSION

This study has demonstrated that acute IH results in significant alterations in several variables associated with energy balance, adipokine release and activation of hypothalamic satiety signals. IH reduced body weight, active cycle food intake, and locomotion. These changes after IH were concomitant with a rise in plasma leptin and a reduction in plasma adiponectin, while adipose tissue had less leptin and more adiponectin protein expression after acute IH exposure. In ARC, acute IH induced phosphorylation of STAT3, reduced total ERK1/2 and phosphorylated ERK1/2, but did not significantly affect Ob-Rb or its negative regulator, SOCS3. Acute IH also induced activation, as determined by immediate early gene product FRA-1/2, of POMC-containing neurons of ARC and increased the protein expression of POMC in ARC. Taken together these suggest that IH induces significant alterations to body energy balance through changes in the secretion of leptin which exerts effects on satiety-inducing pathways within the hypothalamus.

The 8 h exposure to IH reduced body weight compared to Norm animals. However, by the following morning (16 h after IH), body weight change was found not to be different between the two groups. Unexpectedly, this body weight re-gain occurred despite a reduction in overnight food intake. Although the mechanism responsible for this re-gain in body weight is not clear, it is possible that the reduction in active cycle locomotion in IH-exposed animals may have contributed as a result of decreased energy expenditure. The regain of body weight in acute IH-exposed animals may be the result of alterations in

metabolism, as studies in rabbits have suggested that intermittent hypobaric hypoxia may lower circulating levels of thyroid hormones (Sawhney and Malhotra, 1990). Additionally, the reduction in food intake after acute IH was likely associated with the increased plasma leptin concentration, which occurred despite the lack of change in retroperitoneal or epididymal adipose tissue depot mass. It is known that leptin is correlated with adipose tissue content (Maffei et al., 1995). However, it has been reported that under certain pathophysiological challenges this balance can be altered (Kim and Scarpace, 2003). In this study, IH was a sufficient stimulus to elevate plasma leptin concentration and this may have accounted for the overnight reduction in food intake observed in these animals. Therefore, the actions and secretion of leptin appear to be independent of body weight changes induced by IH, while appearing inherently associated with IH. Taken together, these data can be interpreted to indicate that following acute IH, leptin may induce a state of satiety. As previously suggested, this may occur regardless of nutritional status (Levin et al., 1996; Rentsch et al., 1995). This finding is in contrast with reduced plasma concentration of leptin in individuals exposed to altitude-related hypoxia (Woolcott et al., 2002; Zaccaria et al., 2004). However, our findings occur following IH, rather than continuous hypoxia and under normobaric conditions, which may account for this difference. Consistent with this suggestion, it has been reported that IH can increase circulating leptin concentrations in mice (Li et al., 2005). It has been suggested that some IH models can induce sleep deprivation (Gozal et al., 2001). Sleep deprivation has been reported to reduce body weight and energy expenditure

(Vetrivelan et al., 2012), and a reduction (Vetrivelan et al., 2012) or no change (Bodosi et al., 2004) in leptin levels, with no changes in food intake (Barf et al., 2010; Vetrivelan et al., 2012). In humans, sleep deprivation has been shown to increase hunger and reduce leptin levels (Brondel et al., 2010; Spiegel et al., 2004). As well, corticosterone levels in the acute IH-exposed rats was not significantly different from Norm-exposed animals, if IH induced sleep deprivation in these animals, it would be expected that corticosterone levels would higher than controls (Tartar et al., 2009). Taken together, these data would suggest that it was unlikely that sleep deprivation played a significant role in the leptin-feeding responses observed in this study during acute IH.

Adipose tissue leptin content was found to be less in acute IH conditions. This, coupled with the finding of elevated plasma leptin concentrations, suggests an increase in the secretion of the adipokine by adipose tissue. Adipocytes have been demonstrated to increase their secretion of leptin in response to hypoxia (Famulla et al., 2012), and IH caused a sustained hypoxia in adipose tissue (Reinke et al., 2011). Taken together, these findings suggest that adipose tissue hypoxia resulting after acute IH in this study may have increased the release of leptin from adipocytes. Conversely, adiponectin had a lower circulating level, though an increased amount of the protein was found within adipocytes. This is consistent with previous studies showing that hypoxia causes a reduction in adipocyte secretion of the adipokine (Famulla et al., 2012). The changes in circulating levels of adipokines as seen in this study, induces a higher leptin:adiponectin ratio, perhaps as a result of altered adipocyte secretion as a

result of IH. An increase in leptin:adiponectin ratio is a commonly used measure associated with cardiometabolic disease (Satoh et al., 2004; Kotani et al., 2005). The alterations observed in circulating adipokine concentrations, as seen during IH in this study, are consistent with those found in individuals suffering from obesity (Dagogo-Jack et al., 1996) and obstructive sleep apnea (Hargens et al., 2013), of which IH is a major component.

Leptin action in ARC reduces food intake through activation of intracellular signaling pathways associated with Ob-Rb (Peters et al., 2007; Scarpace et al., 2007). The primary pathway associated with leptin's satiety-inducing effects is the phosphorylation of STAT3 in the rat (Ladyman and Grattan, 2004). Homodimerization of pSTAT3 results in translocation of the transcription factor to the nucleus of the affected cell. This induces transcription of several genes, such as *Pomc* and immediate early genes, including *Fra-1* (Bousquet et al., 2000). Our results show that after acute IH, POMC protein content within ARC increases. This finding is supported by the observations of increased *Pomc* mRNA in response to leptin (Mizuno et al., 1998). The effect of leptin on specific neurons within ARC is thought to be dependent on the phenotype of the neurons within this region. Leptin has been shown to increase the firing rate of POMC-containing neurons (Cowley et al., 2001; Wang et al., 2008), while inhibiting the activity of neurons expressing neuropeptide Y (van den Top et al., 2004; Wang et al., 2008). Increased POMC in the hypothalamus induces hypophagia (Zhang et al., 2011), and we have found that acute IH induces both an increase in ARC POMC, as well as hypophagia. Our findings show that the IH leads to activation

of POMC-containing neurons within this region as it was found that a larger number of POMC neurons in ARC expressed the immediate early gene product FRA-1/2. The finding of increased FRA-1/2 expression in cells has been used to indicate alterations in neuronal activity, including depolarization under a variety of conditions, including IH (Messenger et al., 2012). These findings are further supported by the observation of transcriptional upregulation of satiety genes within the hypothalamus following chronic IH (Volgin and Kubin, 2006).

In summary, acute IH results in reductions in body weight, food intake and locomotion, and can alter the release of adipokines that regulate food intake from adipose tissue. Intracellular signaling pathways that are normally associated with satiety signaling were elevated in response to IH and this likely lead to the increased activity of POMC-containing neurons within ARC. These findings suggest that acute IH may result in altered body energy balance possibly through activation of hypothalamic pathways.

2.6 FOOTNOTES

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CHAPTER 3

ACUTE INTERMITTENT HYPOXIA DOES NOT ALTER ENERGY

BALANCE IN THE LEPTIN-DEFICIENT KILO RAT

3.   CHAPTER 3

3.1 CHAPTER SUMMARY

Intermittent hypoxia (IH) is a major pathophysiological consequence of obstructive sleep apnea. Recently it has been described that acute IH results in complex alterations in body energy balance, induced leptin secretion and concomitant alterations in feeding pathways within the hypothalamus. To determine the role of leptin on these changes, leptin-deficient KILO rats were exposed to IH or normoxic control conditions. Body weights, consumatory and locomotor behaviours and hypothalamic alterations were assessed immediately following exposures. IH failed to alter measures of body weight, fat pad mass, food intake, locomotor activity, and the plasma concentration of angiotensin II. Plasma leptin concentration was undetectable by enzyme immunoassay. Within the arcuate nucleus of the hypothalamus, no changes were observed in phosphorylated signal transducer and activator of transcription 3, pro-opiomelanocortin, long- or short-form leptin receptor, suppressor of cytokine signaling 3, nor within phosphorylated extracellular signal-regulated kinase ½. This study suggests that leptin plays an essential role in mediating the alterations observed in body energy balance and hypothalamic activity following IH compared to normoxic controls. Whether this effect is due to a lack of leptin directly acting in the hypothalamus, failure to initiate the release of a secondary factor, or altered chemoreceptor reflex sensitivity is not known.

3.2 INTRODUCTION

Intermittent hypoxia (IH) is a major pathophysiological consequence of obstructive sleep apnea, a condition during which there is the episodic closures of the airway during sleep. A substantial reduction in pulmonary oxygen tension is observed during IH resulting in hypoxemia (Dempsey et al., 2010; Simon and Collop, 2012). As a result of this whole-body hypoxic insult, several physiological systems are altered, including the cardiovascular (Levy et al., 2012; Freet et al., 2013) and respiratory (Debevec and Mekjavic, 2012; Dempsey et al., 2012) systems. It has been shown that alterations to the endocrine system are also induced by both sustained hypoxia and IH (Dempsey et al., 2010; Moreau and Ciriello, 2013; Reinke et al., 2011; Tschop et al., 2000). In particular, studies in both humans and experimental animal models have shown plasma leptin concentration to be elevated following IH (Messenger et al., 2012; Moreau and Ciriello, 2013; Tschop et al., 2000), and this is often associated with alterations in body energy balance (Moreau and Ciriello, 2013; Tschop et al., 1998). Leptin is an important modulator of satiety. Released by white adipocytes, the 16-kDa protein hormone signals arcuate nucleus of the hypothalamus (ARC; Satoh et al., 1997). Within ARC, leptin increases the activity of pro-opiomelanocortin (POMC) neurons, while inhibiting neurons expressing neuropeptide Y (Elmquist et al., 1998). When activated, POMC neurons mediate satiation and anorexigenic responses (Balthasar et al., 2004; Dhillon et al., 2006).

Recently, we have shown that acute IH can alter central mechanisms important for the regulation of body energy balance (Moreau and Ciriello, 2013). This study

demonstrated that leptin, a satiety-inducing hormone, released into the circulation occurred after acute IH. Subsequently, a reduction in food intake was observed, despite an initial loss of body weight. These effects were observed concomitant to the induction of the anorexigenic factors phosphorylated signal transducer and activator of transcription 3 (pSTAT3), pro-opiomelanocortin (POMC), and a reduction of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) within arcuate nucleus of the hypothalamus (Moreau and Ciriello, 2013). Whether leptin played a direct role in these observed effects following IH is not known.

Therefore, this study was done to determine whether the effects of acute IH on body energy balance and associated hypothalamic feeding pathways are initiated as a result of the release of leptin following IH. To determine this, leptin-deficient rats were exposed to IH or normoxic conditions, and assessed for various measures of body weight, behaviors and plasma hormones associated with body energy balance and compared to wild type Sprague Dawley rats (WT). ARC was also removed to determine potential alterations in signaling in hypothalamic feeding pathways.

3.3 METHODS AND MATERIAL

3.3.1 Animals and study design

Adult male, Sprague-Dawley rats (300-350 g; n=12) were obtained from Charles River Canada (Sherbrooke, QC). Adult male, homozygous, leptin-deficient KILO rats (250-400 g; n=8; SD-$Lep^{tm1sage}$) were obtained from Sigma Advanced Genetic Engineering Laboratories (TGRA3780; Vaira et al., 2012). Rats were individually housed at a temperature of 22°C and 60% relative humidity with access to food and water available *ad libitum*, except during the 8 h IH or normoxic exposure, in 12h light/dark cycle conditions. Animals were handled in accordance with the guidelines set forth by the Canadian Council on Animal Care and the Animal Use Committee at the University of Western Ontario.

3.3.2 IH and normoxic exposures

Animals were assigned to either IH or normoxia group for exposure. Following these exposures, physiological measures of body weight and consumatory behaviours were measured. Animals were exposed to IH and normoxic conditions as previously described (Messenger and Ciriello, 2013; Messenger et al., 2012, 2013; Moreau and Ciriello, 2013). In brief, animals were placed in chambers consisting of four tubes (10 cm diameter by 35 cm length) and a zero-pressure escape valve. For IH-exposed animals, a computer that regulated solenoid valves altered the input of N_2 or room air to generate IH conditions.

Animals were exposed to 80s hypoxia (6.5% O_2) followed by 120s normoxia. The levels of O_2 and CO_2 were monitored by sensors on the chamber, which relayed information back to the computer to ensure proper cycling. Conditions within the chamber were isobaric (770 ± 11 mmHg) and eucapnic (<0.1% CO_2) (Moreau and Ciriello, 2013). Normoxic animals were exposed to only the air input.

3.3.3 Measures of body weight, food intake and water intake

Animals were weighed immediately before and after IH or normoxic exposure. These values were used to calculate body weight changes during the exposure period, overnight body weight gain and 24 h body weight change. Furthermore, food and water intake were measured over the remaining 16 h following IH or normoxic exposure (Moreau and Ciriello, 2013).

3.3.4 Locomotion assay

Immediately following IH or normoxic exposure (1700 h), animals were placed in home cages with access to food and water *ad libitum*. Animals were placed into large cages (60 cm x 40 cm) with a floor 4x5 grid system in the dark (red light on) 30 min into the dark (active) cycle (1930 h). Over a 5 min period, the number of crosses of a line by an animal was determined by two independent, blinded observers to determine horizontal locomotion. Simultaneously, vertical locomotion was determined by the number of rearing events. An average value

was then calculated from these two observers for both horizontal and vertical locomotion (Moreau and Ciriello, 2013).

3.3.5 Plasma collection and enzyme immunoassays

As previously described (Moreau and Ciriello, 2013), immediately after the cross-over exposure, rats were immediately sacrificed under equithesin anaesthesia (0.3 ml/100 g b.w.; i.p.). Blood samples were collected by cardiac puncture in the presence of 7% ethylenediaminetetraacetic acid at a volume of 10 µl/ml blood and immediately centrifuged at 10 000 RPM for 10 min at 4 °C to isolate the aqueous plasma. This aqueous plasma phase was removed and analyzed using enzyme immunoassays for rat leptin (Enzo Life Sciences; Farmingdale, NY) and angiotensin II (Phoenix Pharmaceuticals; Burlingame, CA) according to manufacturer instructions. Enzyme immunoassay plates were read on a SpectraMax M5 plate reader using SoftMax Pro v.5 microplate analysis software (Molecular Devices; Sunnyvale, CA).

3.3.6 Tissue collection and preparation

Immediately after exposure to IH or normoxia, rats were sacrificed under equithesin anesthesia (0.3 ml/100 g b.w.; Moreau and Ciriello, 2013) and the brains removed and frozen at -80°C until analyzed. Using a circular micropunch tool (1 mm internal diameter), 500 µm punches of ARC were taken and

immediately homogenized in cold radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate) with protease inhibitor cocktail (Roche Applied Science; Laval, QC) by an electric homogenizer (VWR International; Radnor, PA). Homogenates were sonicated over three passages for 15s each on ice (55%; Sonic Dimembrator Model 150; Fisher Scientific). Samples were then rotated for 10 min at 4 °C and centrifuged at 4°C for 20 min at 14000 RPM. Protein content of homogenates was quantified using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories; Hercules, CA). Protein samples were added to 25% sample buffer and 10% reducing buffer (Life Technologies; Burlington, ON) and water to a standard protein concentration of 1.67 mg/ml (Moreau and Ciriello, 2013; Messenger and Ciriello, 2013).

3.3.7 Western blots

As previously described (Messenger et al., 2012; Moreau and Ciriello, 2013), electrophoresis was carried out using a 10% discontinuous polyacrylamide Bis-Tris gel (Life Technologies; Burlington, ON), followed by standard protein immunoblotting techniques. From each animal, 25 μg of protein of each sample was loaded. Electrophoresis was carried out at 200 V and terminated when the dye front reached the bottom of the gel. Proteins were transferred to a polyvinylidene fluoride membrane using a wet transfer method in the presence of methanol and SDS (50 mM Tris, 40 mM glycine, 0.3% SDS, 20% methanol) and

wet transfer apparatus (Mini Trans-Blot Electrophoretic Transfer Cell; Bio- Rad Laboratories; Hercules, CA) at 100 V for 2 h. After transfer, the membrane was washed in Tris-buffered saline + Tween-20 (TBST; 20 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween-20; pH 8.0) blocked for 1 h with 5% skim milk made in TBST buffer at room temperature. The membrane was then incubated with primary antibodies diluted in skim milk over night at 4 °C. The following day, the membrane was washed with TBST before being incubated with horseradish peroxidase-conjugated secondary antibodies-specific to the appropriate host of the primary antibody being analyzed, for 1 h at room temperature. For detection, the membrane was washed with TBST, followed by distilled water and then stained using a horseradish peroxidase substrate enhanced chemiluminescence system (Luminata Forte, EMD Millipore; Billerica, MA). Blots were visualized using a VersaDoc imaging system (Bio-Rad Laboratories; Hercules, CA) and analyzed using ImageLab v.3.0 (Bio-Rad Laboratories; Hercules, CA).

3.3.8 Antibodies

The following antibodies were used for western blots: rabbit anti-β-actin-HRP (1:50000; A3854, Sigma-Aldrich; St. Louis MO), chicken anti-ObRB (1:5000; CH14104, Neuromics; Edina, MN), rabbit anti-STAT3 (1:2000; #9132, Cell Signaling; Boston, MA), rabbit anti-pSTAT3 (Tyr705) (1:1000; #9131, Cell Signaling; Boston, MA), rabbit anti-POMC (1:2000; RB-08-0013, RayBiotech; Norcross GA), donkey anti-rabbit IgG-HRP (1:10000; 711-035-152, Jackson

Immunoresearch; West Grove, PA), donkey anti-chicken IgY-HRP (1:10000; 703-035-155, Jackson Immunoresearch; West Grove, PA).

3.3.9 Statistics and analysis

For physiological measures and circulating factors, differences between IH and normoxic groups of WT and KILO rats were determined by two-way ANOVA, followed by a Bonferroni post-hoc analysis. Differences between IH and normoxic exposures were determined within WT and KILO groups for western blots using an unpaired, two-tailed Student t-test, and a p-value < 0.05 was taken to indicate statistical significance. All values were expressed as mean ± standard error. All bar charts were made using GraphPad Prism v.5 graphing software (GraphPad Software; La Jolla, CA).

3.4 RESULTS

3.4.1 Food and water intake

In the 16 h following exposure, WT normoxic animals consumed 30.2 ± 2.3 g of food, or 8.6 ± 1.3 g/100 g b.w. whereas WT IH exposed animals consumed less food (21.1 ± 2.1 g, or 5.9 ± 0.5 g of food/100 g b.w.). KILO rats exposed to normoxia consumed 29.9 ± 1.8 g of food, or 9.4 ± 1.6 g /100 g b.w., while IH exposed KILO rats consumed 31.2 ± 2.4 g of food, or 9.6 ± 1.5 g/100 g b.w. For both absolute (Fig 3.1a) and standardized food intakes (Fig 3.1b), WT IH consumed significantly less food than WT normoxic. In the KILO rats, this effect was not observed (Fig. 3.1a-b). There was no significant difference between WT normoxic exposed rats and normoxic and IH exposed KILO rats, though these relationships were complex as both had an interaction. There were no significant differences between any groups for water consumption.

3.4.2 Food conversion efficiency

WT animals exposed to normoxia had a food conversion efficiency of 0.46 ± 0.13 g b.w. gain/g food consumed, whereas IH exposed WT rats had food conversion efficiency of 1.11 ± 0.15 g b.w. gain/g food consumed. Normoxic KILO rats had a food conversion efficiency of 1.00 ± 0.07 g b.w. gain/g food consumed, while KILO rats exposed to IH were 1.05 ± 0.05 g b.w. gain/g food consumed. In WT animals, IH animals had higher food conversion efficiency, while this difference

Figure 3.1 Energy intake and utilization in KILO rats following IH.

Food intake and food conversion efficiency are altered in WT, but not KILO rats exposed to IH. Bar charts indicate total (a) and standardized (b) food intake over 16h immediately following exposure to IH or normoxia in WT and KILO rats. Calculated food conversion efficiency over the same period (c) is also shown. NS: non-significant; * $p < 0.05$ as determined by two-way ANOVA followed by a Bonferroni post-hoc analysis. Data are presented as mean ± standard error. n=6 for WT groups, n=4 for KILO groups.
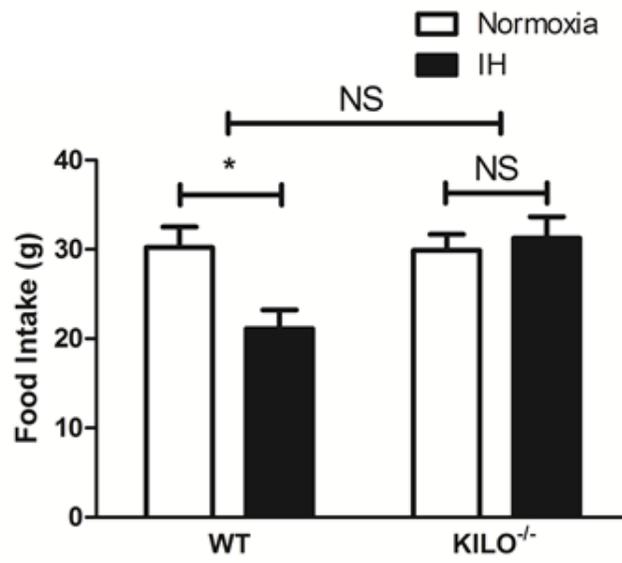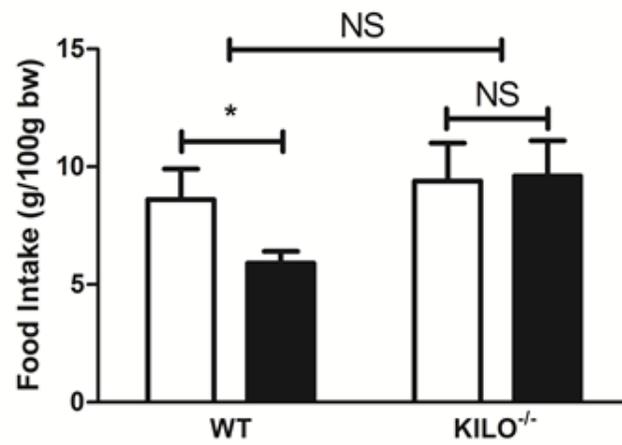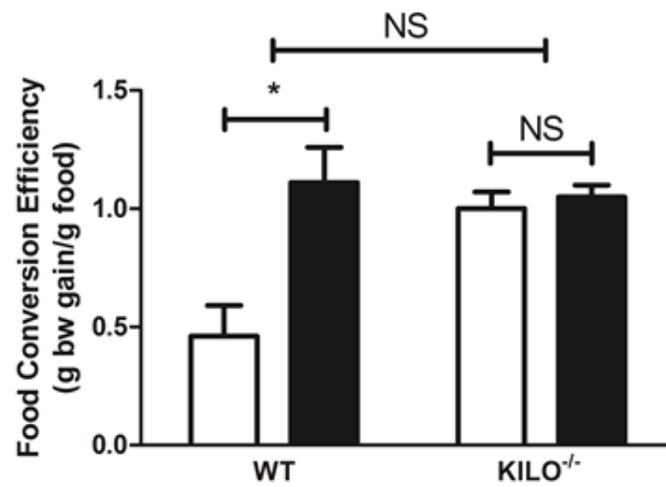
was not observed in leptin-deficient KILO rats. KILO rats did not have significantly different food conversion efficiency from WT, though the comparisons were complex with a significant interaction being present (Fig 3.1c).

3.4.3 Body weight changes

Prior to exposure, there were no significant differences (p = 0.99) in body weights between KILO IH (380 ± 63 g) and KILO normoxic (380 ± 75 g), and WT IH (365 ± 10 g) and WT normoxic (364 ± 9 g) groups. During the exposure to either IH or normoxia, WT IH exposed animals lost approximately 8% of their body weight (-29.3 ± 1.9 g) and WT normoxic animals lost approximately 3% (-13.8 ± 1.2 g) of their body weight. KILO IH animals lost 7% of their body weight (-28.4 ± 1.5 g), and normoxic KILO animals lost about 6% of their body weight (-24.5 ± 2.2 g). Body weight lost during exposure was significantly higher in WT IH animals compared to normoxic controls, though this effect was not observed in KILO animals. There was no significant difference between the body weight lost during exposure between WT and KILO animals, though a significant interaction was found (Fig 3.2a). In the 16 h immediately following the exposures, WT normoxic animals had a regained body weight change of 14.1 ± 3.2 g while IH animals regained 23.6 ± 4.5 g. KILO normoxic animals regained 29.9 ± 2.0 g overnight while the KILO IH animals regained 32.6 ± 2.3 g in the same time period. WT animals exposed to IH gained more body weight overnight, while leptin-deficient KILO rats did not differ in their response to IH compared to normoxia. KILO

animals gained significantly more body weight overnight than WT animals (Fig 3.2b). The overall change in body weight for the 24h following the initiation of exposure was 0.4 ± 3.7 g for WT normoxic, -5.6 ± 2.6 g for WT IH exposed animals, 5.4 ± 3.2 g for normoxic KILO and 4.2 ± 3.0 g for IH KILO animals. There were no significant differences between IH and normoxic exposed animals of either strain though KILO animals gained significantly more body weight compared to WT animals over the same time period (Fig 3.2c).

3.4.4 Fat pad mass

The mass of the epididymal fat pad was 3.1 ± 0.8 g in WT normoxic animals, 2.8 ± 0.6 g in WT IH animals, 15.2 ± 2.6 g in KILO normoxic animals, and 13.2 ± 2.8 g in KILO IH animals. No differences were observed between IH and normoxia exposures in either WT or KILO animals. However, KILO rats did have significantly higher fat pad mass compared to WT animals (Fig 3.3a). The retroperitoneal fat pad mass of WT normoxic animals was 5.2 ± 1.2 g, 4.8 ± 1.2 g in WT IH animals, 24.4 ± 2.9 g in normoxic KILO rats, and 22.2 ± 3.1 g in KILO IH animals. There were no statistical differences between normoxia and IH exposed animals of either genetic background, although KILO rats had significantly larger retroperitoneal fat pads (Fig 3.3b).

Figure 3.2 Body weight in KILO rats exposed to IH.

Body weight is significantly altered following IH in WT, but not KILO rats. Bar charts indicating body weight change during exposure (a), overnight body weight gain (b), and overall body weight change 24h from baseline (c) in WT or KILO animals exposed to normoxia or IH. NS: non-significant; * $p < 0.05$ as determined by two-way ANOVA followed by a Bonferroni post-hoc analysis. Data are presented as mean ± standard error. n=6 for WT groups, n=4 for KILO groups.
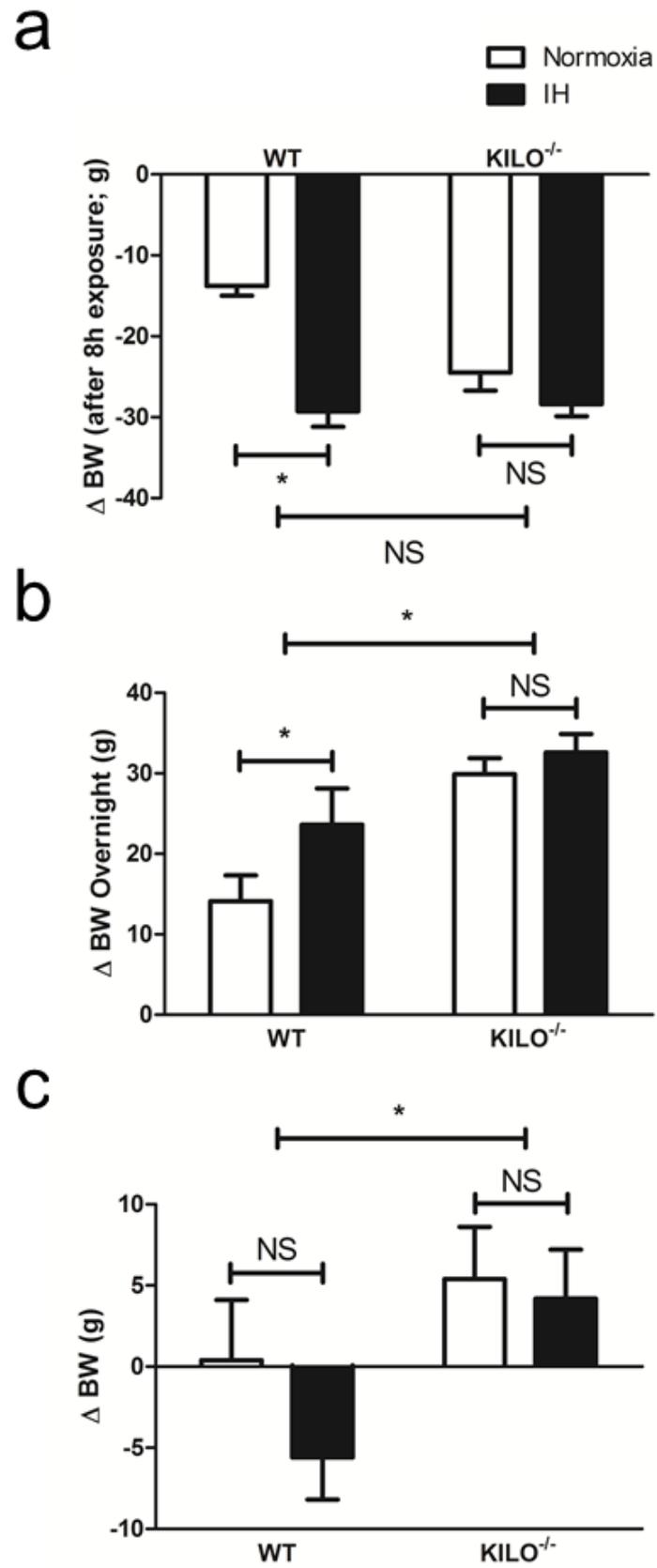
3.4.5 Locomotion

Horizontal locomotor activity in normoxic WT animals was 112 ± 9 line crossings/5 min and in IH WT animals 87 ± 3 line crossings/5 min. In the KILO rats exposed to normoxia, animals had horizontal locomotion of 93 ± 7 line crossings/5 min while the IH KILO rats had 87 ± 8 line crossings/5 min. WT animals exposed to IH had significantly less horizontal locomotor activity. This effect of IH was not found in KILO animals (Fig. 3.3c). There were no significant differences between WT and KILO animals, though an interaction was observed (Fig 3.3c).

Vertical locomotor activity was measured in normoxic WT animals to be 31 ± 3 rearings/5 min, 28 ± 4 rearings/5 min in IH WT animals, 26 ± 4 rearings/5 min in normoxic KILO animals and 23 ± 4 rearings/5 min in IH KILO animals. There were no significant differences between or among the groups for vertical locomotor activity (Fig 3.3d).

3.4.6 Plasma leptin concentration

WT rats exposed to normoxia had a circulating leptin level of 3.8 ± 1.0 ng/ml, while WT rats had significantly elevated plasma leptin concentrations of 11.1 ± 1.3 ng/ml following IH exposure. KILO rats exposed to normoxia had leptin concentration of 0.2 ± 0.1 ng/ml and IH exposed KILO rats had a similar plasma level (0.2 ± 0.1 ng/ml). KILO animals had a significantly lower concentration of

Figure 3.3 Fat pad mass and locomotion in KILO rats following IH.

Fat pad mass and locomotion in WT and KILO animals exposed to either normoxia or IH. Bar charts indicating epididymal (a) and retroperitoneal (b) fat pad mass in either WT or KILO animals following either normoxia or IH. Horizontal (c) and vertical (d) locomotor activities in in WT and KILO animals exposed to either normoxia or IH are also displayed. NS: non-significant; * $p < 0.05$ as determined by two-way ANOVA followed by a Bonferroni post-hoc analysis. Data are presented as mean ± standard error. n=6 for WT groups, n=4 for KILO groups.

a Epididymal Fat Mass (g) — WT vs KILO⁻/⁻, Normoxia and IH. NS between WT groups; NS between KILO⁻/⁻ groups; * between WT and KILO⁻/⁻.

b Retroperitoneal Fat Mass (g) — WT vs KILO⁻/⁻, Normoxia and IH. NS between WT groups; NS between KILO⁻/⁻ groups; * between WT and KILO⁻/⁻.

c Horizontal Movement (Line Crossings/5 min) — WT vs KILO⁻/⁻, Normoxia and IH. * between WT groups; NS between KILO⁻/⁻ groups; NS between WT and KILO⁻/⁻.

d Vertical Movement (Rearings/5 min) — WT vs KILO⁻/⁻, Normoxia and IH. NS between WT groups; NS between KILO⁻/⁻ groups; NS between WT and KILO⁻/⁻.
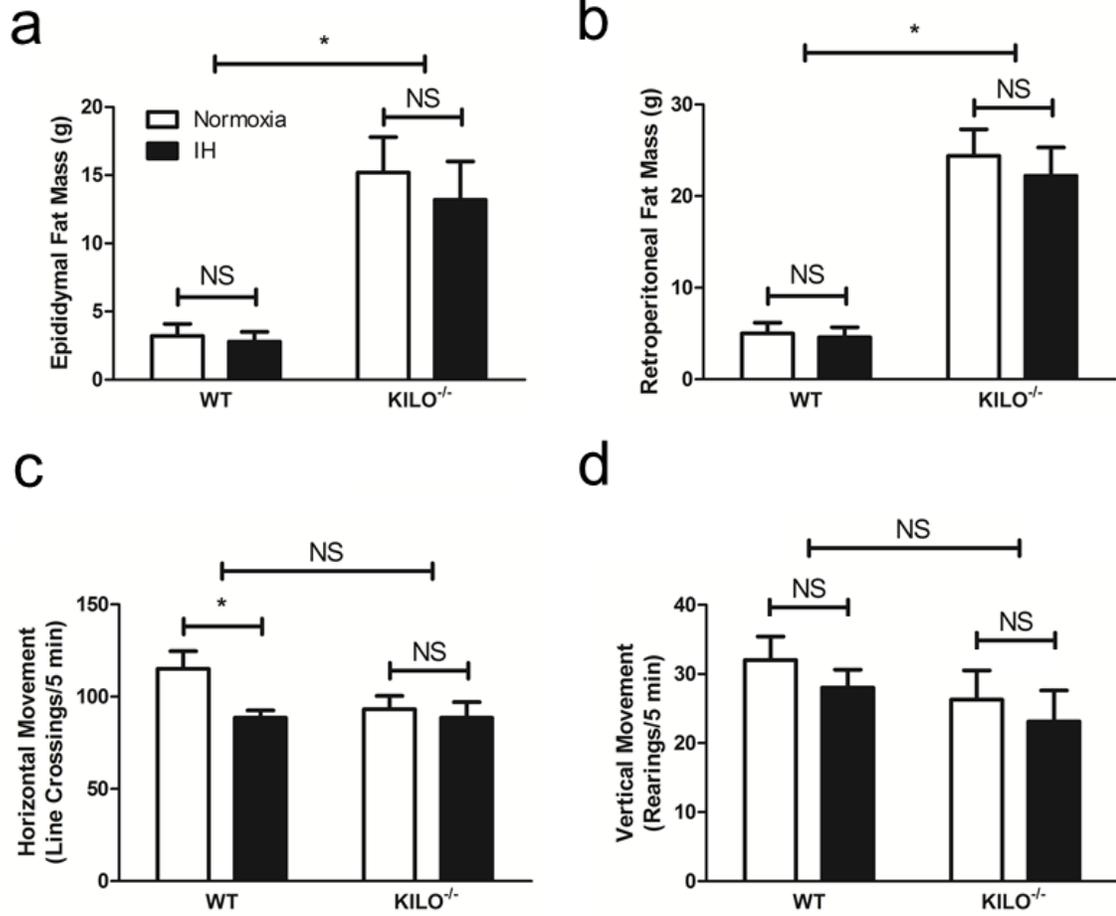
leptin than WT animals, regardless of exposure, and there was an interaction between both exposure and genetic background of the animals (Fig 3.4a).

3.4.7 Plasma angiotensin II concentration

WT normoxic animals had an angiotensin II plasma concentration of 0.65 ± 0.20 ng/ml. WT IH animals had a greater than 2 fold increase in circulating level of angiotensin II (1.55 ± 0.17 ng/ml). KILO rats exposed to normoxia had a circulating angiotensin II concentration of 0.91 ± 0.10 ng/ml. Similarly, KILO rats exposed to IH had a circulating plasma angiotensin II concentration of 0.87 ± 0.10 ng/ml (Fig 3.4b).

3.4.8 ARC leptin signaling molecules

Within ARC, the amount of ObRB protein was not different between normoxic and IH exposed animals for either WT (p = 0.65; Fig 3.5a) or KILO animals (p = 0.90; Fig 3.5b). The amount of β-actin protein was not different between normoxia exposed and IH exposed WT (p = 0.88) or KILO animals (p = 0.68).

IH WT animals had significantly more (p = 0.0031) pSTAT3 compared to normoxic WT animals (0.78 ± 0.03 A.U. vs. 0.12 ± 0.02 A.U., respectively; Fig 3.5c). This effect was not observed within the ARC of KILO animals (p = 0.72) exposed to normoxia (0.0056 ± 0.0007 A.U.) or IH (0.0060 ± 0.0008 A.U.; Fig

Figure 3.4 Plasma hormone concentrations in KILO rats exposed to IH.

Plasma concentration of leptin and angiotensin II in WT and KILO animals in response to either IH or normoxia. Bar charts indicate the plasma concentration of leptin (a) and angiotensin II (b) in WT and KILO animals exposed to either IH or normoxia. NS: non-significant; * $p < 0.05$ as determined by two-way ANOVA followed by a Bonferroni post-hoc analysis. Data are presented as mean ± standard error. n=6 for WT groups, n=4 for KILO groups.
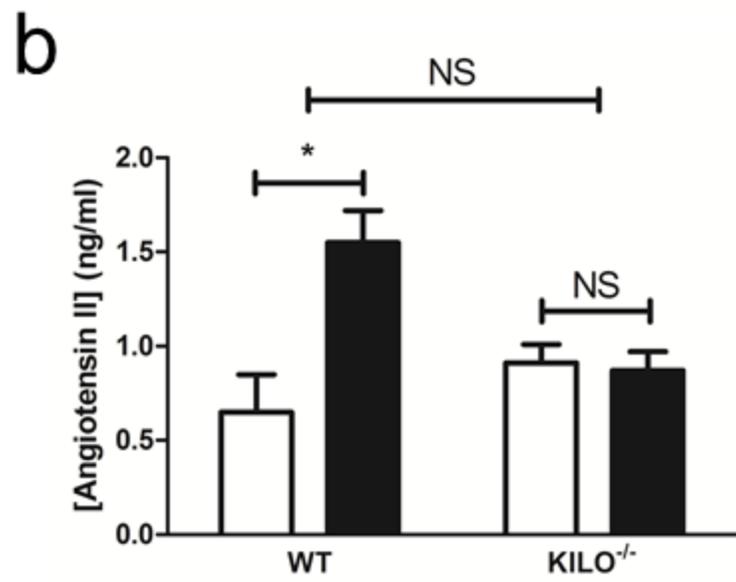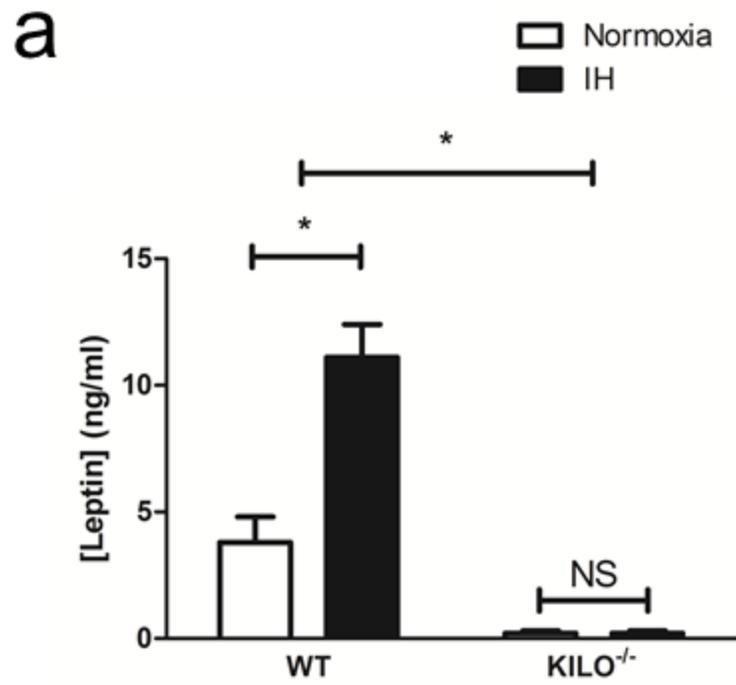
3.5d). The total amount of STAT3 protein was not different for either WT (p = 0.84) or KILO (p = 0.70) animals.

POMC protein levels were significantly higher (p = 0.022) in ARC of WT animals exposed to IH (1.10 ± 0.31 A.U.) compared to normoxia (0.51 ± 0.15 A.U.; Fig 3.5e). No significant difference (p = 0.43) was observed in POMC protein levels within ARC of KILO rats exposed to normoxia (0.034 ± 0.0043 A.U.) or IH (0.029 ± 0.003 A.U.; Fig 3.5f).

Figure 3.5 Leptin signaling molecules in ARC of KILO rats exposed to IH.

Representative blots and bar graphs depicting important leptin signaling molecules in ARC of WT and KILO rats. ObRB/β-actin within the ARC of WT (a) and KILO rats (b). Note that the amount of ObRB to β-actin protein within the ARC is not changed in WT or the KILO rat. (c-d), representative blots and bar graphs depicting pSTAT3/STAT3 within ARC of WT (c) and KILO rats (d). Note that the amount of pSTAT3 to total STAT3 protein within the ARC is elevated in WT, but not the KILO rat. (e-f), representative blots and bar graphs depicting POMC/β-actin within ARC of WT (e) and KILO rats (f). Note that the amount of POMC to β-actin protein within the ARC is elevated in WT, but not the KILO rat. *, $p < 0.05$ as determined by unpaired, two-tailed t-test. Data are presented as mean ± standard error. n=6 for WT groups, n=4 for KILO groups.

a ObRB WT

b ObRB KILO⁻/⁻

c pSTAT3 WT

d pSTAT3 KILO⁻/⁻

e POMC WT

f POMC KILO⁻/⁻
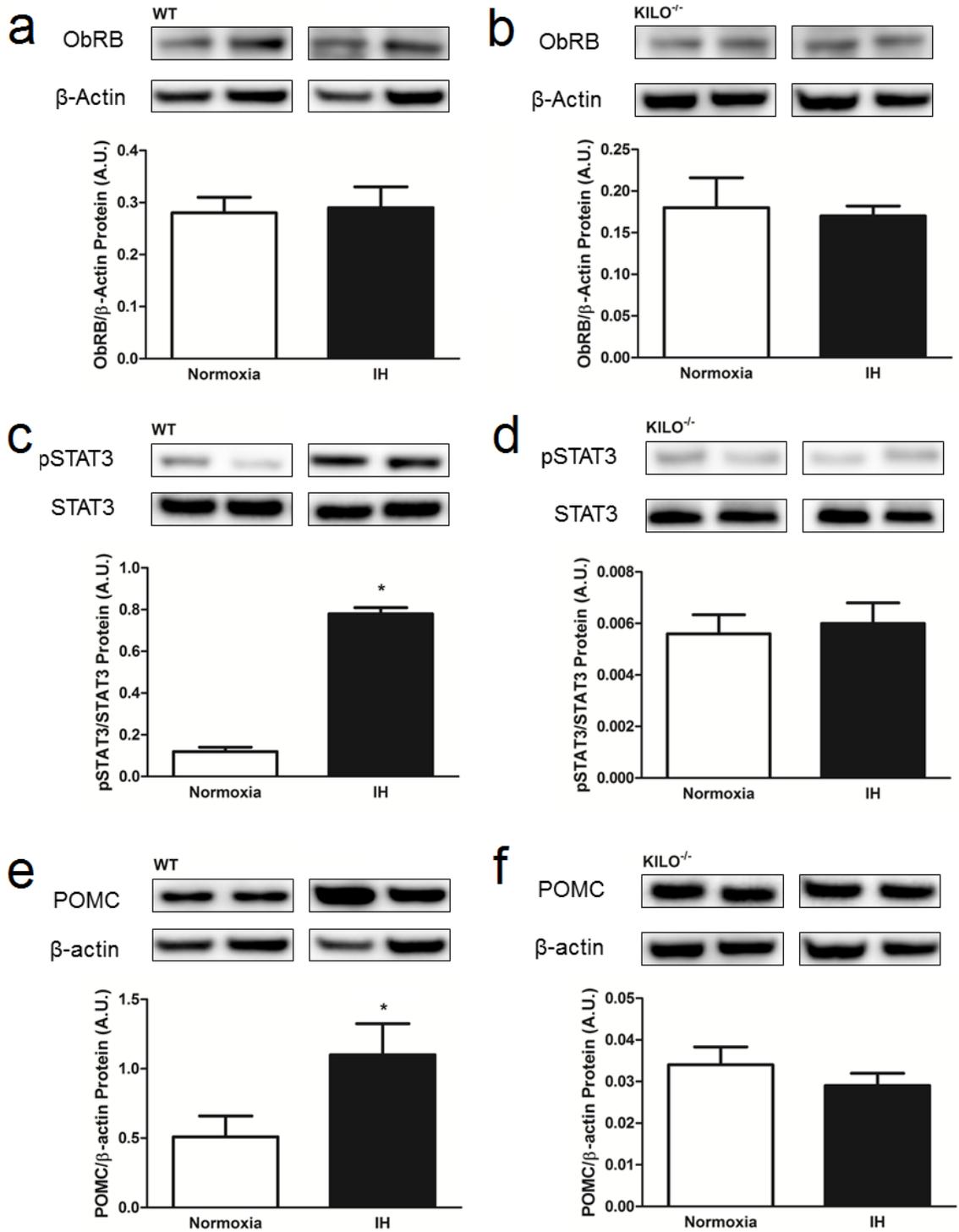
3.5 DISCUSSION

This study has shown that the effects of IH previously described (Moreau and Ciriello, 2013) on body energy balance, hypothalamic signaling, and circulating hormones are mediated by the leptin released in responses to IH as the effects were not present in the leptin-deficient KILO rat. This is based on the findings that IH in WT animals reduced body weight during exposure, and increases overnight regain of the body weight. This effect is concomitant with a reduction in food intake and food conversion efficiency, effects not observed in the KILO rat. Additionally, elevated circulating angiotensin II and leptin are observed following IH exposure in WT animals, but these effects were not observed in KILO animals, suggesting that the absence of leptin in response to IH in KILO rats is important in the release of angiotensin II into the circulation in response to IH. Finally, in ARC, activation of downstream pSTAT3 and increased POMC were found following IH in WT animals. However, these differences are not observed in ARC of the leptin-deficient KILO animal. Taken together, these findings suggest that leptin released into the circulation is essentially for body energy balance and hypothalamic signaling in response to IH.

In an earlier study, significant reduction in body weight during 8h IH and a subsequent significant increase in body weight regain in the 16 h immediately following exposure has been described (Moreau and Ciriello 2013). As a result, the 24 h body weight change was not different between IH and normoxic exposed animals. As previously argued (Moreau and Ciriello, 2013), the loss of body weight due to exposure of IH is likely not due to a reduction in specific

adipose depot tissue mass, although it may be a cumulative effect from all sources of adipose tissue. Following chronic IH, a reduction in adipose tissue mass and body weight has also been observed (Martinez et al., 2008). Given that leptin deficient animals exposed to IH failed to lose more body weight during 8h IH suggests that the mechanisms resulting in weight loss during IH are dependent on the presence of leptin released into the circulation during IH (Messenger et al., 2012; Moreau and Ciriello, 2013).

Circulating leptin can reduce food intake by acting on neurons within ARC (Satoh et al., 1997). Once bound to the ObRB, leptin causes autophosphorylation of JAK2 and subsequent homodimerization of pSTAT3 (Banks et al., 2000; Kloek et al., 2002; White et al., 1997). pSTAT3 then acts to regulate transcription of various genes, including *Pomc* (Banks et al., 2000). After acute IH, plasma leptin concentrations were increased and increased levels of pSTAT3 and POMC were observed in ARC as previously described (Moreau and Ciriello, 2013). In the absence of leptin, as in the present study, these changes in anorexigenic signaling molecules are not altered in response to IH. This suggests the activation of satiety signals in ARC due to IH is dependent upon the presence of leptin. This effect may not be direct, as other systems have been shown to increase activation of pSTAT3 and POMC within ARC (Anderson et al., 2003; Zhang et al., 2011). However, if they were responsible for the observed changes, they must ultimately fall under the control of leptin, as these changes are abolished in the leptin-deficient KILO rat.

An increase in circulating angiotensin II concentration was also observed in WT animals following IH. Unexpectedly, this effect was abolished in the KILO rat. This suggests that the release of angiotensin II following IH is dependent on leptin signaling. The mechanism by which leptin may contribute to the elevation of angiotensin II is not known, but may involve actions of leptin in the brainstem (Ciriello, 2013) or at the level of the carotid body (Messenger et al., 2012) to increase peripheral chemoreceptor sensitivity (Ciriello and Moreau, 2013), which in turn may increase renal sympathetic nerve activity (Ciriello, 2013), a known stimulus for the renin-angiotensin hormonal pathway (Froeschl et al., 2013).

In conclusion, this study has shown that leptin plays an essential role in mediating the effects of acute IH on changes to body energy balance, and related hormonal secretion and molecular alterations within ARC. Taken together, these data suggest that leptin may be an essential mediator of the physiological response to hypoxia.

3.6 FOOTNOTES

3.7 REFERENCES

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CHAPTER 4

CHRONIC INTERMITTENT HYPOXIA INDUCES LEPTIN

RESISTANCE

4.   CHAPTER 4

4.1 CHAPTER SUMMARY

Obstructive sleep apnea (OSA) is strongly correlated with the presence of obesity and it has been determined that patients with OSA experience weight gain, in addition to elevated leptin concentrations. Chronic intermittent hypoxia (CIH) is a major pathophysiological manifestation of OSA, and acute IH has recently been shown to alter body energy balance. Therefore, this study set out to determine the relationship of body energy balance and leptin signaling in CIH. To examine this, Sprague-Dawley rats were exposed to 95 days of CIH or normoxic control conditions, and were assessed for daily measures of body weight, food intake, and food conversion efficiency. Towards the end of the study, leptin sensitivity, locomotor activity and circulating leptin was determined within animals. Following the study period, fat pads and arcuate hypothalamic nuclei (ARC) were isolated. ARC were assessed for factors associated with leptin signaling. CIH animals increased their food intake over the study period, whereas normoxic animals reduced this value. Normoxic animals had a reduced daily body weight change over the study period, whereas this value was not different in CIH animals. Epididymal fat mass and food conversion efficiency was less in CIH animals. Following peripheral leptin injection, normoxic animals reduced food intake, whereas CIH animals did not. Basal concentrations of leptin were not different between the groups, but were elevated in CIH when normalized for epididymal fat mass. Within ARC, CIH animals had elevated SOCS3 protein.

This study suggests that CIH, as seen in OSA, can induce a state of leptin resistance.

4.2 INTRODUCTION

Obesity is an energy balance disorder that affects a large number of individuals within society (Ogden et al., 2013). This disease is associated with a positive energy balance, whereby individuals do not utilize as many calories as consumed throughout the day. The caloric imbalance observed in obesity is believed to be the result of leptin resistance, which is considered to be the primary risk factor for both obesity and overweight (Morris et al., 2010). During leptin resistance, the satiety and anorexigenic effects of leptin are lost, thus promoting dysregulation of caloric consumption, preventing negative feedback onto energy storage sites such as the adipose tissue (Kalra et al., 1998). As a result, hyperphagia persists, resulting in an increased deposition of adipose tissue mass (Lin et al., 2000). The resultant increase in adipose tissue causes an elevation in circulating leptin concentrations, potentially furthering the resistance to leptin (Scarpace et al., 2002).

Hyperphagia and obesity are caused by a reduced expression of leptin signaling molecules such as the long-form leptin receptor (ObRB) in the ARC (Gong et al., 2008). Once leptin binds ObRB, phosphorylation and homodimerization of signal transducer and activator of transcription 3 (STAT3) occurs (Vaisse et al., 1996). Activation by phosphorylation of extracellular regulated kinase 1/2 (ERK1/2) also occurs (Banks et al., 2000). The translocation of pSTAT3 to the nucleus induces the transcription and production of pro-opiomelanocortin (POMC), a major effector for satiety, and suppressor of cytokine signaling 3 (SOCS3; Banks et al., 2000; Munzberg et al., 2003; Bates et

al., 2003), a negative regulator of ObRB (Bjorbak et al., 2000). Another important negative regulator of leptin signaling is protein tyrosine phosphatase 1B (PTP1B; Sahu, 2004). Leptin resistance is associated with a decrease in activity of leptin within the arcuate nucleus of the hypothalamus (ARC; Ladyman and Grattan, 2004; Munzberg et al., 2004). The mechanisms that drive leptin resistance are not known, but may occur at the blood-brain barrier leptin transport system, such as the short forms of the leptin receptor (ObR100; Banks et al., 1999), at the level of the receptor (Rahmouni et al., 2008) or post-receptor signaling (Cheng et al., 2002; Mori et al., 2004), including changes in the down-stream melanocortin system (Marsh et al., 1999).

Obesity is a major risk factor for the development of obstructive sleep apnea (Peppard et al., 2000), which is a sleep-related breathing disorder characterized by nocturnal intermittent hypoxia. Patients with obstructive sleep apnea have elevated leptin levels, and a greater disposition to weight gain (Phillips et al., 2000). Recently, we have demonstrated that acute intermittent hypoxia alters energy balance, increases plasma leptin levels and activates leptin-related signaling mechanisms within ARC (Moreau and Ciriello, 2013). Taken together, these results would suggest that chronic intermittent hypoxia (CIH) may induce a state of leptin resistance. To test this possibility, experiments were done in which leptin resistance was determined by the effects of acute leptin injections on food intake, altered energy balance, and concomitant alterations in ARC after exposure to long term CIH.

4.3 METHODS AND MATERIAL

4.3.1 Animals

Experiments were done in adult male Sprague-Dawley rats (360-400 g) individually housed in a room maintained at a temperature of 22°C and 60% relative humidity. All animals had access to food and water *ad libitum*, except during the 8h of intermittent hypoxic or normoxic exposure, in 12h light/dark cycle conditions. Animals were randomly assigned to either CIH (n=8) or normoxia (n=8) groups and exposed to the corresponding conditions for 95 days. Animals were handled in accordance with the guidelines set forth by the Canadian Council on Animal Care and Use Committee at the University of Western Ontario.

4.3.2 CIH and normoxic exposures

Animals were exposed to CIH and normoxic conditions as previously described (Moreau and Ciriello, 2013; Messenger and Ciriello, 2013; Messenger et al., 2012, 2013). Briefly, animals were placed in chambers each consisting of four tubes (10 cm diameter by 35 cm length) and a zero-pressure escape valve. For CIH-exposed animals, a computer that regulated solenoid valves altered the input of N_2 or room air to generate CIH conditions. Animals were exposed to 80s hypoxia (6.5% O_2) followed by 120s normoxia. The levels of O_2 and CO_2 were monitored by sensors on the chamber, which relayed information back to the

computer to ensure proper cycling. Conditions within the chamber were isobaric (770 ± 11 mmHg) and eucapnic (<0.1% CO_2). Normoxic animals were exposed to only the air input.

4.3.3 Measurement of body weight, food and water intake

Each day throughout the study, animals were weighed immediately before and after CIH or normoxic exposure. These values were used to calculate body weight changes during the exposure period, overnight body weight gain and 24h body weight change. Additionally, food and water were measured over the 16h immediately following CIH or normoxic exposure on each day.

4.3.4 Locomotion assay

Immediately following CIH or normoxic exposure on days 91 and 92, animals were returned to their home cages and were allowed access to food and water *ad libitum*. Thirty min into the dark (active) cycle (1930 h), animals were placed into large cages (60 cm x 40 cm) with a floor 4x5 grid system in the dark (red light on). Over a 10 min period, the number of crosses of a line by an animal was determined by two independent observers blinded to their exposure regime to determine horizontal locomotion (Moreau and Ciriello, 2013). Simultaneously, vertical locomotion was determined by the number of rearing events. An average

value was then calculated from these two observers for both horizontal and vertical locomotion (Moreau and Ciriello, 2013).

4.3.5 Leptin resistance assay

Following CIH or normoxic exposure on days 93 and 94, animals were placed into their home cages with *ad libitum* access to food and water. At the beginning of the dark cycle (1900 h), animals were randomly assigned to vehicle or leptin groups, and injected (i.p.) with either 0.4 mg/kg carrier-free recombinant rat leptin (598-LP; R&D Systems, Minneapolis, MN) dissolved to 1 mg/ml in 20 mM Tris HCl (pH = 8.0), or vehicle. Food intake measurements were taken at 1, 2, 3 and 14h after the leptin injection. Animals were exposed to CIH or normoxic conditions the next day, and then were crossed-over to be injected with opposite injectate (i.e. vehicle instead of leptin and vice versa), such that each animal received an injection of both the vehicle and leptin for comparisons.

4.3.6 Plasma collection and enzyme immunoassays

The day following exposure, animals were immediately sacrificed under equithesin anesthesia (0.3 ml/100 g b.w.; i.p.) (Moreau and Ciriello, 2013). Blood samples were collected by cardiac puncture in the presence of 7% ethylenediaminetetraacetic acid at a volume of 10 μl/ml blood. This blood was immediately centrifuged at 10 000 RPM for 10 min at 4°C to isolate the aqueous

plasma. This aqueous plasma phase was removed and stored frozen at -80 °C until analyzed for hormone content. Plasma samples were analyzed using enzyme immunoassay for rat leptin (Enzo Life Sciences; Farmingdale, NY) according to manufacturer instructions. Enzyme immunoassay plates were read on a SpectraMax M5 plate reader using SoftMax Pro v.5 microplate analysis software (Molecular Devices; Sunnyvale, CA).

4.3.7 Tissue collection and preparation

Following exposure to CIH or normoxia, animals were sacrificed under equithesin anesthesia and the brains immediately removed and frozen at -80°C. Using a circular 1 mm (internal diameter) micropunch tool, 500 μm punch-outs of ARC were taken and immediately homogenized in cold radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate) with protease inhibitor cocktail (Roche Applied Science; Laval, QC) by an electric homogenizer (VWR International; Radnor, PA). Homogenates were then sonicated over three passages for 15s each on ice (55%; Sonic Dimembrator Model 150; Fisher Scientific). Samples were then rotated for 10 min at 4°C and centrifuged at 4°C for 20 min at 14000 RPM. Protein content of homogenates was quantified using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories; Hercules, CA). Protein samples were added to 25% sample buffer and 10% reducing buffer (Life Technologies; Burlington, ON)

and water to a standard protein concentration of 1.67 mg/ml (Messenger and Ciriello, 2013; Moreau and Ciriello, 2013).

4.3.8 Western blots

Electrophoresis was carried out using a 10% discontinuous polyacrylamide Bis-Tris gel (Life Technologies; Burlington, ON), followed by standard protein immunoblotting techniques (Messenger et al., 2013; Moreau and Ciriello 2013). For each animal, 25 µg of protein of each sample was loaded. Electrophoresis was carried out at 200 V and terminated when the dye front reached the bottom of the gel. Proteins were transferred to a polyvinylidene fluoride membrane using a wet transfer method in the presence of methanol and SDS (50 mM Tris, 40 mM glycine, 0.3% SDS, 20% methanol) and wet transfer apparatus (Mini Trans-Blot Electrophoretic Transfer Cell; Bio- Rad Laboratories; Hercules, CA) at 100 V for 2h. After transfer, the membrane was washed in Tris-buffered saline + Tween-20 (TBST; 20 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween-20; pH 8.0) blocked for 1 h with 5% skim milk made in TBST buffer at room temperature. The membrane was then incubated with primary antibodies diluted in skim milk over night at 4°C. The following day, the membrane was washed with TBST before being incubated with horseradish peroxidase-conjugated secondary antibodies-specific to the appropriate host of the primary antibody being analyzed, for 1h at room temperature. For detection, the membrane was washed with TBST, followed by distilled water and then stained using a horseradish peroxidase

substrate enhanced chemiluminescence system (Luminata Forte, EMD Millipore; Billerica, MA). Blots were visualized using a VersaDoc imaging system (Bio-Rad Laboratories; Hercules, CA) and analyzed using ImageLab v.3.0 (Bio-Rad Laboratories; Hercules, CA).

4.3.9 Antibodies

For western blots the following antibodies were used: rabbit anti-β-actin-HRP (1:50000; A3854, Sigma-Aldrich; St. Louis MO), rabbit anti-ObR (1:1000; OBR12-A, Alpha Diagnostics International; San Antonio, TX) chicken anti-ObRB (1:5000; CH14104, Neuromics; Edina, MN), rabbit anti-STAT3 (1:2000; #9132, Cell Signaling; Boston, MA), rabbit anti-pSTAT3 (Tyr705) (1:1000; #9131, Cell Signaling; Boston, MA), rabbit anti-ERK1/2 (1:2000; #9102, Cell Signaling; Boston, MA), rabbit anti-pERK1/2 (Thr202/Tyr204) (1:1000; #9101, Cell Signaling; Boston, MA), rabbit anti-POMC (1:2000; RB-08-0013, RayBiotech; Norcross GA), rabbit anti-SOCS3 (1:1000; ab16030, Abcam; Cambridge, MA), goat anti-PTP1B (1:500; sc-1718, Santa Cruz Biotechnology; Dallas, TX), donkey anti-rabbit IgG-HRP (1:10000; 711-035-152, Jackson Immunoresearch; West Grove, PA), donkey anti-chicken IgY-HRP (1:10000; 703-035-155, Jackson Immunoresearch; West Grove, PA), donkey anti-goat IgG-HRP (1:10000; 705-035-003, Jackson Immunoresearch; West Grove, PA).

4.3.10 Statistics and analysis

Differences between and within CIH and normoxic groups at week 1 and week 13 of physiological variables, as well within basal plasma leptin concentrations of fasted and non-fasted animals, were determined by two-way ANOVA with a repeated measure between the exposure time groups, followed by Bonferroni post-hoc analysis. Western blot analyses and fat pad mass between CIH and normoxic groups were compared using an unpaired, two-tailed t-test. For leptin resistance assays, a paired two-tailed t-test was used to compare vehicle injection to leptin injection within CIH and normoxic groups. For all statistical analyses, a p-value < 0.05 was taken to indicate statistical significance. All values are expressed as mean ± standard error. All charts were made using GraphPad Prism v.5 graphing software (GraphPad Software; La Jolla, CA).

4.4 RESULTS

4.4.1 Body weight changes

Prior to exposure, body weights between CIH (385.5 ± 9.9 g) and normoxic (386.5 ± 10.5 g) groups were not significant different (p = 0.95). Average body weight after the first week CIH exposure was 386 ± 11.3 g, while the normoxic animals weighed an average of 402.4 ± 9.6 g. After the thirteenth week of exposure, CIH animals weighed 584.8 ± 12.2 g and normoxic animals weighed 635.6 ± 21.8 (Fig 4.1a). There was both a significant effect of the duration of exposure and exposure type on body weight (Fig. 4.1a-d). After the first week of exposure, CIH exposed animals lost on average approximately 6% of their body weight (-23.7 ± 1.9 g), whereas normoxic controls lost about 3% of their body weight (-12.6 ± 0.7 g; Fig. 4.1b). After the thirteenth week of exposure, CIH animals lost an average of 5% of body weight (-31.8 ± 3.2 g) and normoxic animals lost about 2% of body weight during exposure (-11.9 ± 2.2 g; Fig 4.1b). There was a significant effect of exposure between CIH and normoxic groups. In addition, the CIH group lost significantly more body weight on average after 13 weeks of exposure compared to the first week, but no effect of exposure length on normoxic animals was observed. In the 16h following exposure after the first week, CIH animals gained an average of 25.9 ± 1.5 g and normoxic animals gained 18.0 ± 0.6 g. After 13 weeks, CIH animals gained an average of 32.0 ± 3.2 g and normoxic animals gained 12.8 ± 2.0 g overnight (Fig 4.1c). CIH animals gained significantly more weight overnight than normoxic animals, and this overnight body weight gain increased over the length of exposure. On the other

hand, normoxic animals had decreased overnight body weight gain over the same time period. Average daily body weight change after the first week for CIH animals was 2.1 ± 0.5 g, while normoxic animals gained an average of 5.5 ± 0.4 g. After 13 weeks, CIH animals had an average daily body weight change of -1.1 ± 0.8 g, while normoxic animals had 0.46 ± 0.77 g (Fig 4.1d). There was a significant effect of exposure on daily body weight change, with CIH animals gaining less weight daily. The effect of exposure length on daily body weight change is complex as normoxic exposed animals reduce their daily body weight change, while CIH animals do not significantly alter this response.

4.4.2 Fat pad mass changes

Epididymal fat pad mass was significantly reduced (p = 0.024) in CIH exposed animals (6.1 ± 1.1 g) compared to those exposed to normoxia (10.1 ± 0.8 g) (Fig. 4.1e). Although no differences (p = 0.17) were observed in retroperitoneal fat pad mass between CIH (8.4 ± 1.7 g) and normoxic animals (12.0 ± 1.8 g), a trend towards a decrease was also observed (Fig. 4.1f). These effects were observed even when accounting for total body weight. Epididymal fat of CIH animals (1.08 ± 0.14 g/100 g b.w.), was significantly less (p = 0.028) than normoxic animals (1.60 ± 0.11 g/100 g b.w.). Retroperitoneal fat was not different (p = 0.15) between CIH (1.48 ± 0.23 g/100 g b.w.) and normoxic animals (1.89 ± 0.24 g /100 g b.w.).

Figure 4.1 Effect of CIH on body weight changes and fat pad mass.

Bar charts summarizing the changes body weight (a), body weight change during 8h IH exposure (b), 16 h post-exposure overnight body weight gain (c), and daily change in body weight between animals exposed to CIH or normoxia averaged for over week 1 and week 13 (d). Bar charts summarize epididymal (e) and retroperitoneal (f) fat pad mass after 13 weeks of CIH or normoxia exposure. Note that body weight, body weight changes and adipose tissue mass are altered by CIH. Data are shown as mean ± standard error. *, $p < 0.05$ (determined by two-way ANOVA followed by a Bonferroni post-hoc analysis for body weights, and as determined by an unpaired, two-tailed Student's t-test for fat pad mass). n = 8 per group.
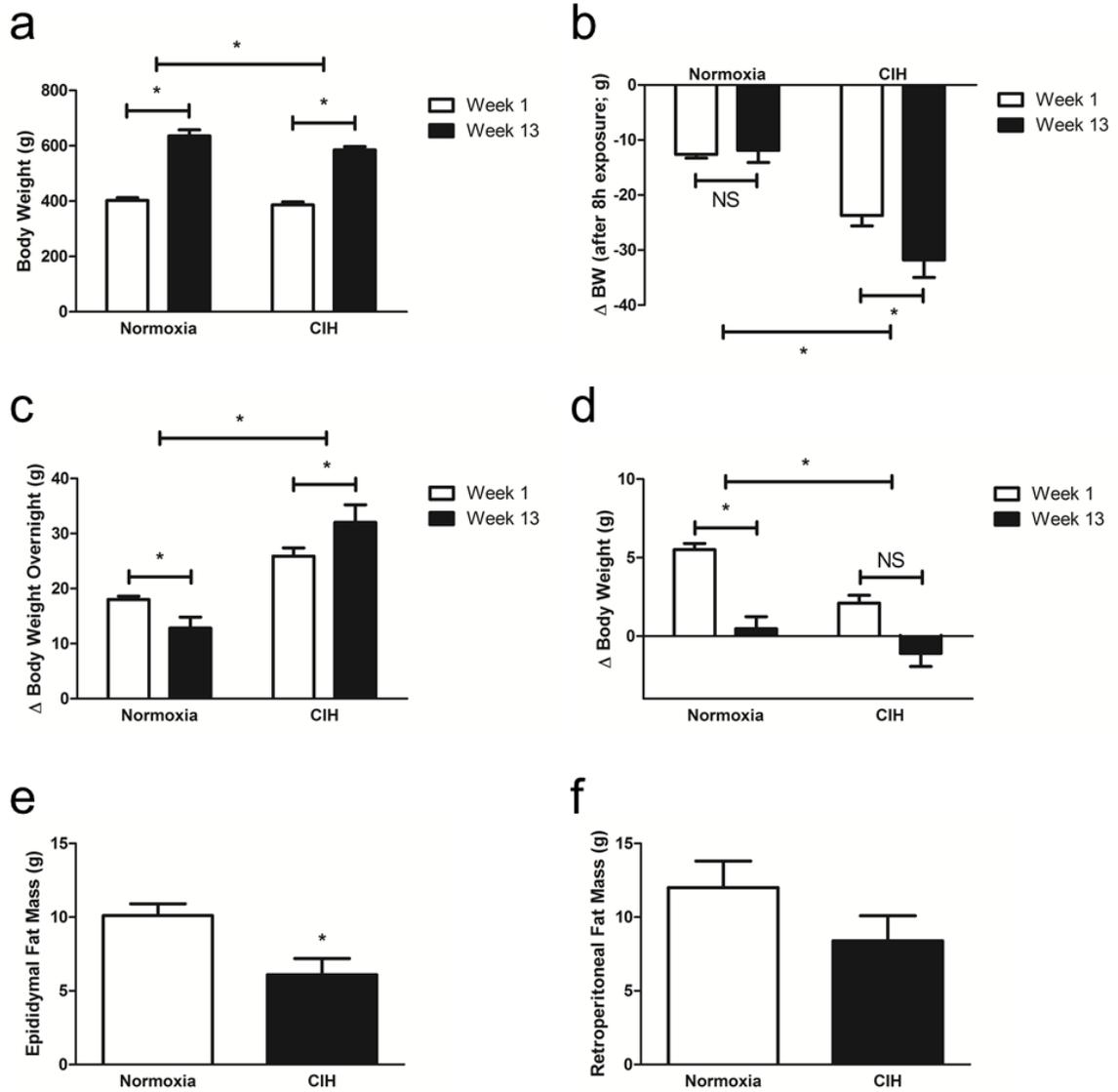
4.4.3 Food intake

In the 16h following daily exposure after the first week, CIH animals consumed an average of 25.2 ± 1.0 g of food while the normoxic animals consumed 31.4 ± 0.8 g of food (Fig. 4.2a). Following 13 weeks of exposure, CIH animals consumed a daily average of 27.5 ± 0.7 g of food, representing an increase of approximately 9%, and normoxic animals consumed 27.9 ± 1.1 g of food, representing a decrease of approximately 11% (Fig 4.2a).

4.4.4 Food conversion efficiency

Food conversion efficiency was 1.04 ± 0.08 g b.w. gain/g food in CIH exposed animals and 0.59 ± 0.03 g b.w. gain/g food in normoxic animals after the first week. After week 13, CIH animals had a food conversion efficiency of 1.08 ± 0.04 g b.w. gain/g food, and normoxic animals were 0.44 ± 0.04 g b.w. gain/g food (Fig 4.2b). There was an effect of exposure for CIH to increase food conversion efficiency, while the relationship of exposure length and exposure interacted to produce a complex relationship whereby there was no alteration within CIH animals over the exposure time, but significantly reduced food conversion efficiency in normoxic animals over the time period.

4.4.5 Locomotion changes

Horizontal locomotor activity was not different (p = 0.66) between CIH and normoxic control rats (301.2 ± 25.8 line crosses/10 min and 295.6 ± 26.1 line crosses/10 min, respectively; Fig 4.2c). Vertical locomotor activity was not different (p = 0.25) between CIH and normoxic rats (83.5 ± 15.8 rears/10 min and 72.4 ± 12.3 rears/10 min, respectively; Fig 4.2d).

4.4.6 Plasma leptin concentrations

Plasma concentrations of leptin during fasting conditions following 95 days CIH had 1.2 ng/ml of leptin in their plasma, while normoxia animals had 1.5 ± 0.3 ng/ml of leptin. When these plasma levels are normalized to epididymal fat content in each animal, CIH exposed animals had a significantly elevated (p = 0.036) plasma leptin than normoxic controls (0.41 ± 0.08 ng/ml/g fat compared to 0.23 ± 0.06 ng/ml/g fat, respectively; Fig 4.2e). Furthermore, a similar finding is observed if the plasma leptin levels are compared to both epididymal and retroperitoneal fat pads.

4.4.7 Leptin resistance induced by CIH

In animals exposed to normoxia, food intake 1 h after acute leptin injection was significantly (p = 0.012) reduced compared to vehicle injected controls (Fig.

Figure 4.2 Changes in food intake and energy expenditure following CIH.

Bar charts showing food intake (a) and food conversion efficiency (b) over the 16h after 1 week or 13 week exposure to CIH or normoxia. In addition, (c-d) show that both horizontal (c) and vertical (d) locomotion were not changed by CIH at the 13 week time-point. Bar chart (e) also shows plasma leptin levels normalized for epididymal fat pad mass. Note that CIH causes changes in food intake and body energy utilization without changing locomotor activity. Furthermore, basal plasma leptin levels are elevated when fat pad mass is taken into account. Data are shown as mean ± standard error. *, $p<0.05$ (determined by two-way ANOVA followed by a Bonferroni post-hoc analysis for food intake, food conversion efficiency, leptin concentration, and as determined by an unpaired, two-tailed Student's t-test for locomotor activities and normalized leptin) n = 4 per group.

a

Food Intake (g)

*

*

*

Week 1
Week 13

Normoxia   CIH

b

Food Conversion Efficiency
(g bw gain/g food)

*

*

NS

Week 1
Week 13

Normoxia   CIH

c

Horizontal Movement
(Line Crossings/10 min)

Normoxia   CIH

d

Vertical Movement
(Rearings/10 min)

Normoxia   CIH

e

[Leptin]$_{plasma}$/Fat
(ng/ml/g Fat)

*

Normoxia   CIH
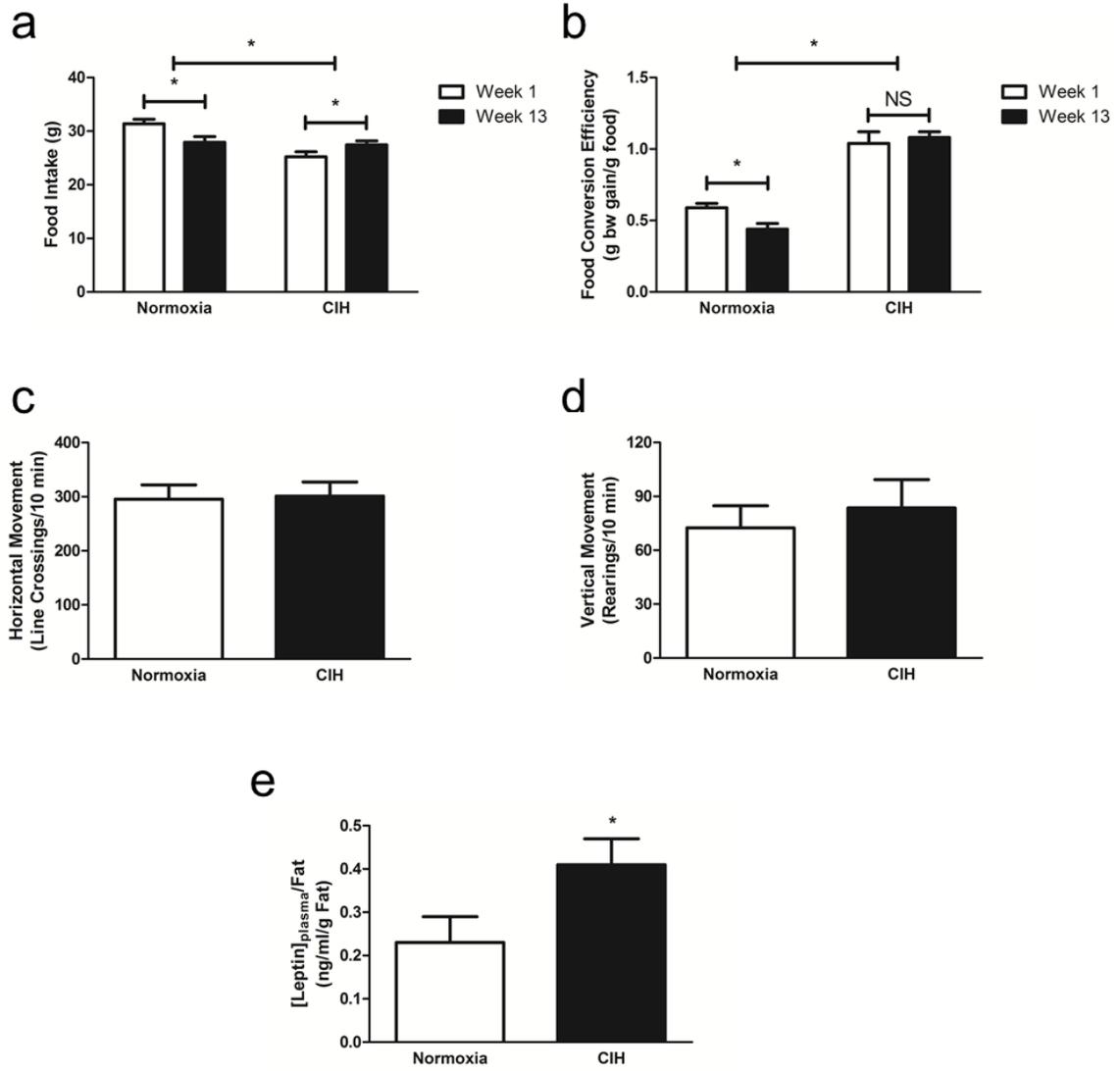
4.3a). On the other hand, in the CIH exposed animals, food intake was not altered at this time point (Fig. 4.3a). Similarly, at 2h and 3h after acute leptin injection, food intake was significantly (2h, $p = 0.0092$; 3h, $p = 0.015$) reduced after acute leptin injections in the normoxic controls compared to vehicle injected animals, but was not altered in the CIH exposed animals (Fig. 4.3b-c). These values were not altered at the 14h time point (Fig. 4.3d). Furthermore, neither vehicle, nor leptin injections in animals exposed to CIH or normoxia altered food intake from total food intake values over the 16h measured on a daily basis.

4.4.8 Protein expression of leptin receptors after CIH

As shown in Figure 4.4, within ARC, CIH did not alter ($p = 0.85$) the protein expression of the long-form leptin receptor (ObRB) compared to normoxia (0.17 ± 0.02 A.U. and 0.16 ± 0.02 A.U., respectively; Fig 4.4a). IH also did not alter ($p = 0.46$) protein expression of the short form leptin receptor ObR_{100} compared to normoxic exposed animals (0.22 ± 0.03 A.U. and 0.24 ± 0.01 A.U., respectively; Fig 4.4b).

4.4.9 Proteins associated with leptin signaling within ARC

Within the ARC, CIH did not alter pSTAT3 protein expression as a function of total STAT3 ($p = 0.46$) compared to normoxic controls (0.15 ± 0.02 A.U. and 0.13 ± 0.02 A.U., respectively; Fig 4.5a), and did not change total STAT3 protein

Figure 4.3 CIH induces resistance to leptin

Bar charts showing cumulative food intake at 1h (a), 2h (b), 3h (c) and 14h (d) post-injection (i.p.) of either 0.4 mg/kg leptin or vehicle treatment. Note that leptin induces satiety in normoxic animals, but not in the CIH exposed animals. Data are presented as mean ± standard error. *, $p < 0.05$ (determined by a paired, two-tailed Student's t-test between vehicle and leptin treatments). n = 8 per group and injection.

a   1 h Post-Injection

b   2 h Post-Injection

c   3 h Post-Injection

d   14 h Post-Injection
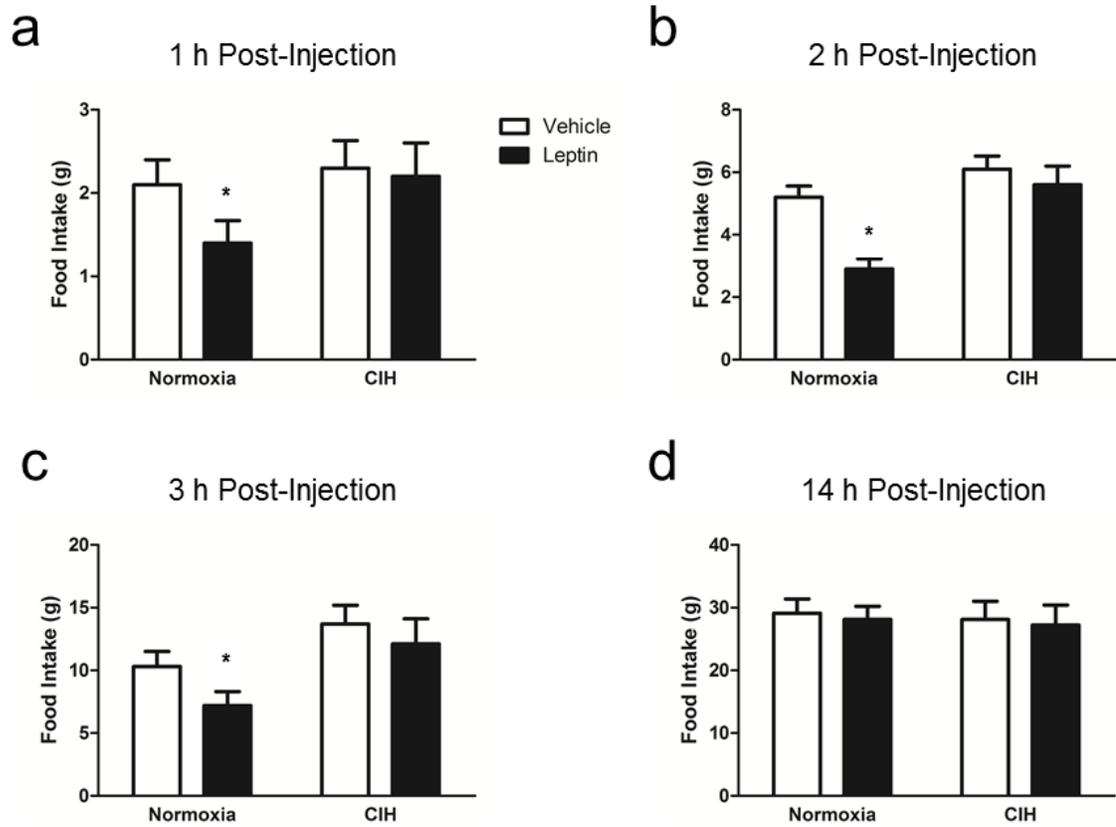
Figure 4.4 Effect of CIH on ObR in ARC

Representative blots and bar graphs depicting ObRB/β-actin protein expression (a) and ObR100/β-actin protein expression (b). Note that leptin receptor isoforms are not altered in ARC following CIH exposure. Data are shown as mean ± standard error. n = 8 per group.

a ObRB

b ObR100
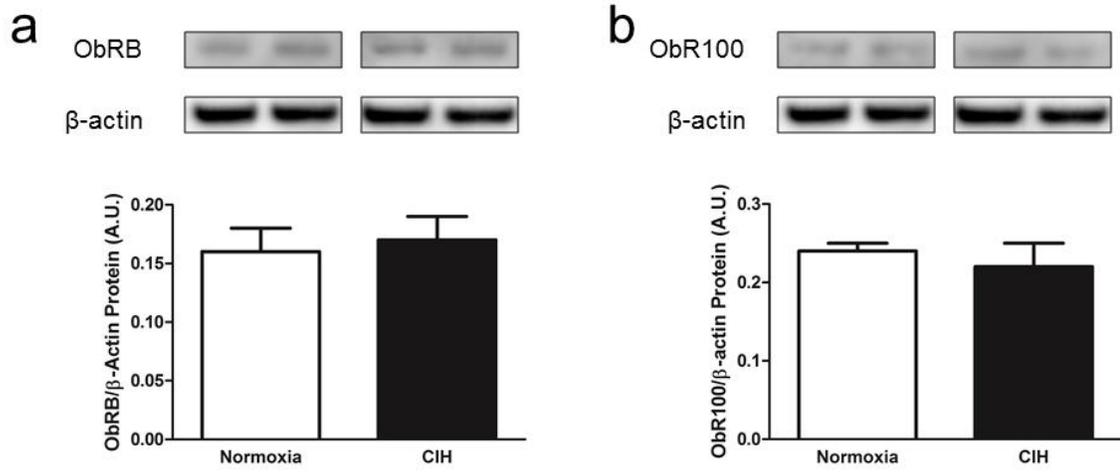
expression (p = 0.39). Protein expression levels of POMC were found to be significantly less (p = 0.045) in CIH (0.41 ± 0.02 A.U.) exposed animals compared to normoxia animals (0.53 ± 0.06 A.U.; Fig 4.5b). Protein expression of total ERK1/2 was significantly less (p = 0.0045) in CIH animals (5.72 ± 0.34 A.U.) compared to ARC of normoxia animals (7.62 ± 0.38 A.U.; Fig 4.5c). However, a significant increase (p = 0.001) in the proportion of ERK1/2 that was phosphorylated was observed in CIH animals (0.12 ± 0.01 A.U.) compared to normoxic control animals (0.043 ± 0.010 A.U.; Fig 4.5d).

4.4.10 Protein markers associated with leptin resistance within ARC

Within ARC, CIH significantly increased (p = 0.022) the expression of SOCS3 protein compared to normoxic controls (0.20 ± 0.03 A.U. and 0.12 ± 0.01 A.U., respectively; Fig 4.6a). On the other hand, no significant difference (p = 0.56) in PTP1B protein expression was observed between CIH (0.038 ± 0.004 A.U.) and normoxic animals (0.041 ± 0.005 A.U.; Fig 4.6b).

Figure 4.5 Leptin signaling proteins in ARC following CIH

Representative blots and bar charts depicting (a) pSTAT3/STAT3 protein expression, (b) POMC protein expression, (c) total ERK1/2 protein expression, and (d) pERK1/2 / ERK1/2 protein expression in ARC. Note that CIH alters leptin signaling protein expression within ARC. Data are presented as mean ± standard error. *, $p < 0.05$ (determined by an unpaired, two-tailed Student's t-test). n = 8 per group.
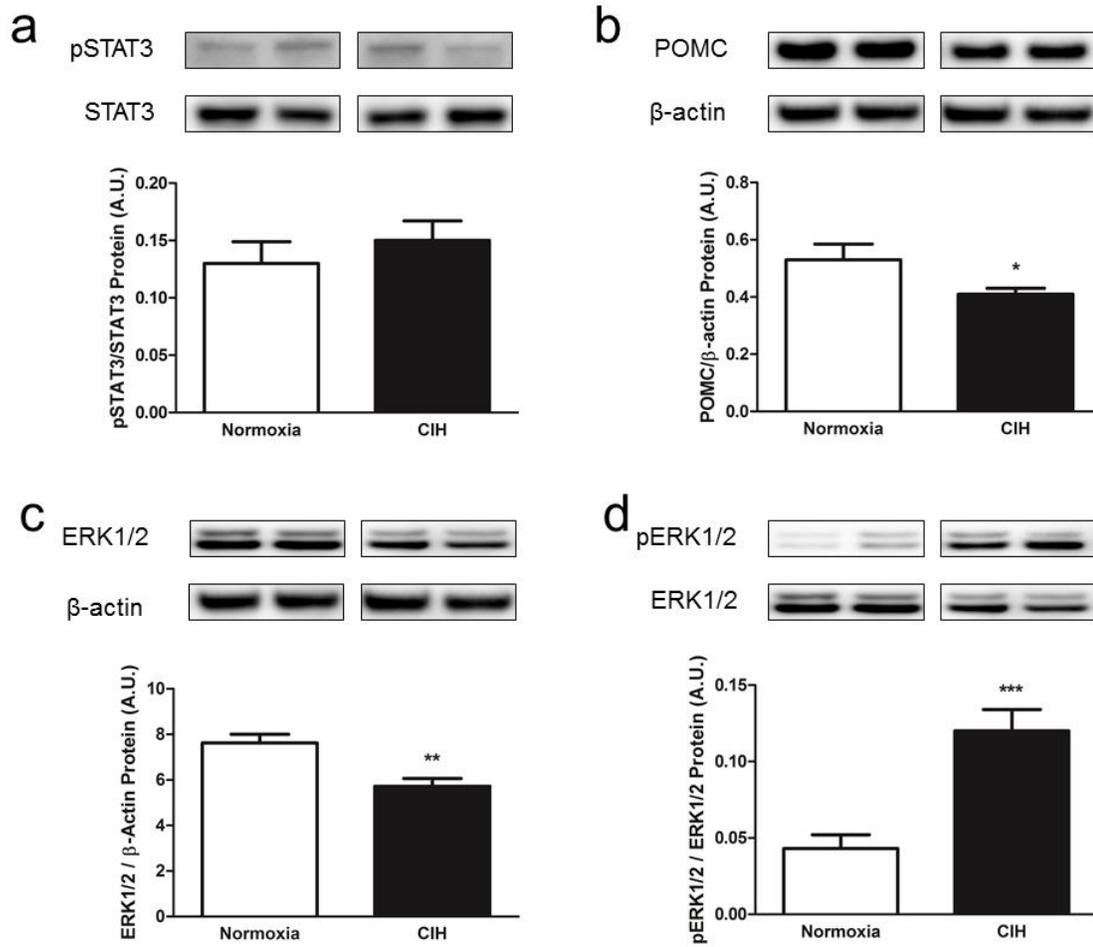
Figure 4.6 Effect of CIH on negative regulators of leptin signaling in ARC.

Representative blots and bar charts depicting inhibitors of leptin signaling SOCS3 (a) and PTP1B (b) protein expressions within ARC. Note that CIH alters SOCS3, but not PTP1B protein expression in the ARC after 13 weeks exposure. Data are presented as mean ± standard error. *, $p < 0.05$ (determined by an unpaired, two-tailed Student's t-test). n = 8 per group.

a SOCS3

β-actin

b PTP1B

β-actin
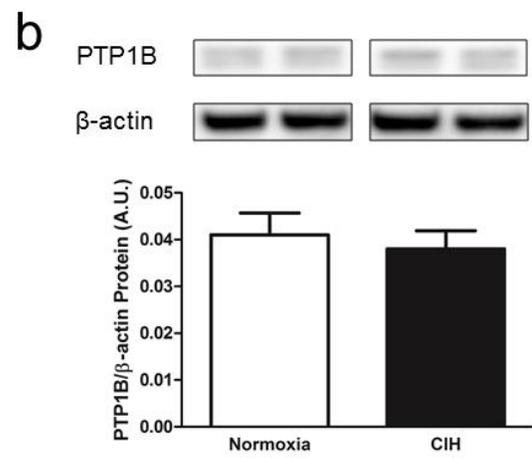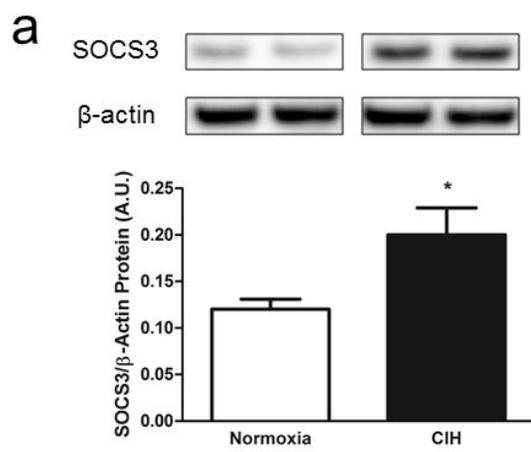
4.5 DISCUSSION

This study has demonstrated that 13 weeks of continuous exposure to intermittent hypoxia induces a state of leptin resistance. Animals exposed to CIH have increased food intake over the study period, where normoxic controls reduce their level of consumption over the same period. This occurs alongside a significant reduction in daily body weight change by controls, while no change is observed in CIH animals. Epididymal fat pad mass is significantly less at the end of the study in CIH compared to normoxic animals. Direct measure of the effect of leptin on food intake indicated a state of leptin resistance in CIH animals, while normoxic animals responded normally to acute administration of leptin. Basal plasma leptin concentrations normalized to epididymal fat mass were was significantly higher in CIH exposed animals compared to normoxic controls. These changes are concomitant with a lower protein amount of ERK1/2 and POMC in ARC, while pERK1/2 and SOCS3 were elevated between CIH and normoxic groups.

In a recent study, we have described the acute effect of intermittent hypoxia on body energy balance (Moreau and Ciriello, 2013). Each bout of exposure to intermittent hypoxia induced a loss of body weight, which was abrogated following non-hypoxic conditions as a result of reduced locomotion and altered body energy utilization. In this study, after 13 weeks of CIH, body weight loss during exposure increases, as opposed to no difference observed within the normoxic animals. It is likely that this effect could was due to a potentiation of the peripheral chemoreceptor reflex on sympathetic flow,

independent of a change in respiration (Prabhakar et al., 2005, 2009; Dick et al., 2007; Xing and Pilowsky, 2010). This conclusion is supported by the finding that CIH has long-term effects on sympathetic outflow (Zoccal et al., 2007, 2008). Given the observed reduction of epididymal fat pad mass, it may be suggested that the break-down of adipose tissue contributes to the body weight loss, a finding also reported by others (Martinez et al., 2008; Drager et al., 2011), although this may not be the primary mediator of the decreased body weight observed following acute intermittent hypoxia (Moreau and Ciriello, 2013).

Overnight food intake was observed in animals exposed to CIH during the first week compared to normoxia. This acute effect was also observed following only 8h of exposure to intermittent hypoxia (Moreau and Ciriello, 2013) and has been reported in humans exposed to chronic hypoxia (Tschop et al., 1998). However, by the thirteenth week food intake between CIH and normoxic animals was not significantly different. In fact, food intake over this time course was reduced in normoxic controls, but was increased in CIH exposed animals. The increased food intake could be suggestive in the CIH exposed animals of a potential manifestation of leptin resistance (Kalra et al., 1998; Trujillo et al., 2011). Similarly, food conversion efficiency, an indirect measure of whole-body energy utilization, was reduced in normoxic animals over the exposure period. This is suggestive of increased body energy utilization. This change was not observed in CIH animals, suggesting their body energy metabolism was not changing in a way concurrent to normoxic control animals. Along the exposure length in the CIH animals, food conversion efficiency was elevated compared to

normoxic animals, suggestive of reduced body energy utilization. Although the long-term effects that food conversion efficiency will have on pre-disposition to weight gain is not well understood, a reduction in metabolic function is associated with weight gain in humans (Houmard, 2008). Whether the lack of increase in body energy utilization is due to leptin resistance in CIH animals is not known, but it is possible that given that leptin augments metabolic activities mediated in part by the sympathetic nervous system (Harris, 2013), and plasma leptin levels are elevated following a bout of intermittent hypoxia (Moreau and Ciriello, 2013), CIH may contribute to leptin resistance. In support of this suggestion, leptin resistance was measured directly by a repeated comparison to leptin and vehicle treatment in CIH and normoxia exposed animals. It was found that in normoxic animals, acute leptin injections caused a significant reduction in food intake in the first three hours. However, in the CIH exposed animals, leptin did not induce a satiating effect when compared to vehicle injections.

Leptin resistance is followed by the development of weight gain and hyperphagia (Kalra et al., 1998; Trujillo et al., 2011). Due to the complex interaction of CIH and this leptin resistant state on body weight, it may be suggested that the effects on body weight may not yet have taken hold in these CIH exposed animals for a long enough period to induce a significant weight gain as recently reported (Guo et al., 2013). An alternate explanation may be that in this model, due to its anorexigenic effects through increased sympathetic activity, an increase in body weight may not be easily demonstrated until animals are exposed to longer periods of CIH, despite evidence that CIH causes a wide

range of metabolic pathophysiologies including dyslipidemia, high blood pressure and insulin resistance (Polotsky et al., 2003; Zoccal et al., 2007; Drager et al., 2011). It should also be kept in mind that there are individuals that suffer from the metabolic syndrome that are not obese, and CIH, as a result of obstructive sleep apnea, can worsen their metabolic profile (Kono et al., 2007).

Another major effect of leptin resistance is chronic hyperleptinemia. Fasting leptin concentrations in the CIH animals were not different compared to normoxic controls. However, given that CIH animals had less body weight and epididymal fat pad mass, it suggested that the observed levels of leptin in the CIH animals were in fact elevated compared to normoxic controls. When fat mass was taken into account, it was found that CIH animals had significantly higher plasma leptin concentrations than their normoxic counterparts. It should be noted that the epididymal fat mass is thought to be the largest producer of leptin in the rat (Zheng et al., 1996). Previous reports by us (Messenger et al., 2012; Moreau and Ciriello, 2013) and others (Polotsky et al., 2003; Li et al., 2006) have shown that acute short-term intermittent hypoxia can increase plasma leptin concentrations. This elevated leptin release immediately following bouts of intermittent hypoxia on a daily basis may contribute to the development of the leptin resistance observed in the 13 week exposed CIH animals.

Following CIH, within ARC, no alterations were observed in the protein expression of either the long- or short form leptin receptors. While the precise mechanism surrounding leptin resistance is not understood, it has been suggested that a reduction in ObRB may prevent appropriate leptin signaling

(Schwartz et al., 1997). From these data, it appears that this may not be the mechanism driving the leptin resistance in this model. Similar suggestions have been made regarding ObR_{100}, where a reduction in this protein would prevent sufficient transport of leptin across the blood-brain barrier (Banks et al., 2001). CIH animals were found not to have altered ObR_{100} protein expression in ARC. However, it should be kept in mind that these do not take into account changes in affinity for the receptor (Mooradian et al., 2000), or changes in receptor localization (Gan et al., 2012).

Within ARC, it was observed that CIH animals had less POMC protein expression than normoxic control animals. Consistent with this finding, a reduction in POMC within ARC has been shown to be associated with hyperphagia (Richard et al., 2011). This could be the result of reduced signaling in leptin resistance, or could function as a mediator of the resistance itself. Also within ARC, SOCS3 protein was found to be higher in CIH than normoxic animals. An increase in SOCS3 would prevent activation of the leptin receptor signaling cascade (Bjorbak et al., 2000), and preventing downstream activation of factors associated with the effect of leptin, such as POMC (Banks et al., 2000). SOCS3 overexpression has previously been shown to occur in leptin resistant states, and experimentally can increase food intake (Bjørbaek et al., 1998; Reed et al., 2010).

Taken together, these data have demonstrated for the first time that exposure to CIH can induce a state of leptin resistance. This pathophysiological state is associated with increased energy balance. Furthermore, this leptin

resistant state is associated with an increased protein expression of SOCS3 and with a concomitant reduction in POMC protein expression within ARC. These findings suggest a possible link between patients exposed to CIH, such as obstructive sleep apnea and the development of obesity and metabolic disorders.

4.6 FOOTNOTES

4.7 REFERENCES

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CHAPTER 5

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INTERMITTENT HYPOXIA INDUCES CHANGES IN PROTEIN EXPRESSION OF NEUROPLASTICITY MARKERS IN THE NUCLEUS OF THE SOLITARY TRACT

5.   CHAPTER 5

5.1 CHAPTER SUMMARY

Intermittent hypoxia (IH) has been shown to alter the response of neurons in nucleus of the solitary tract (NTS) to cardiovascular inputs. The mechanisms involved in these effects may involve pre- and/or post-synaptic neuroplasticity alterations in NTS. To investigate this possibility, Sprague-Dawley wild-type rats were exposed to 1, 7 or 95 days of IH or normoxia. Additionally, to determine whether leptin exerted an effect on the 1 day changes to IH, leptin-deficient (KILO) rats were exposed to IH or normoxia. Arterial pressure (AP) was also measured at these time intervals in conscious wild-type animals. Additionally, at each time point protein was extracted from NTS and analyzed by western blot for the expression of brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TrkB), synaptophysin and growth-associated protein-43 (GAP-43). AP was not different between the IH and normoxic animals at 1 or 7 days. However, after 95 days of IH, AP was significantly elevated compared to normoxic controls. After 1 day IH, TrkB protein expression in NTS was higher while synaptophysin was lower than normoxic wild-type rats. No changes were found in TrkB protein expression in KILO rats after 1 day IH, although synaptophysin protein expression was decreased. BDNF and GAP 43 protein expression levels were not altered in any group following 1 day IH or normoxia. After 7 days of IH, BDNF and TrkB protein expression was found to be elevated in NTS compared to normoxic controls. After 95 days of IH expression of BDNF, synaptophysin, and GAP-43 proteins were less abundant in NTS than in normoxic controls. These

results suggest that neuroplasticity changes occurring within NTS may be

associated with the development of autonomic dysregulation often seen in

patients with chronic obstructive sleep apnea, including elevated blood pressure.

5.2 INTRODUCTION

Obstructive sleep apnea is a chronic, progressive disease that is characterized by episodic closures of the upper airway during sleep (Dempsey et al., 2010). One of the physiological consequences of obstructive sleep apnea is intermittent hypoxia (IH; Freet et al., 2013). IH has previously been shown to activate peripheral carotid chemoreceptors (Cooper et al., 2005). These specialized cells respond to lowered blood oxygen tension and in response increase afferent neural activity to the nucleus of the solitary tract (NTS), the primary site of peripheral chemoreceptor afferent termination (Ciriello et al 1994). It has previously been shown that chronic intermittent activation of the carotid chemoreceptor pathway can result in sympathetic long-term facilitation (Dick et al., 2007; Xing and Pilowsky, 2010), which plays an important role in the exaggerated sympathetic responses seen by further chemoreceptor activation (Xing and Pilowsky 2010).

IH may also induce alterations in the sensitivity of the baroreceptor reflex (Bonsignore et al., 2002; Carlson et al., 1996). Intermittent apneas have been shown to reset the baroreceptor reflex to a higher level (Monahan et al., 2006; Prabhakar and Khumar, 2010) suggesting a possible interaction between activation of the chemoreceptor reflex and a depression of the baroreceptor reflex (Cooper et al., 2005; Somers et al., 1991) during IH. Although the central mechanisms that may induce sympathetic long-term facilitation and baroreceptor reflex depression are not known, they may be important in the development and maintenance of hypertension observed in individuals suffering from chronic

obstructive sleep apnea. We have recently demonstrated that IH can increase plasma leptin concentration (Messenger et al., 2012; Moreau and Ciriello, 2013). Circulating leptin has been shown to potentiate the chemoreceptor reflex (Ciriello and Moreau, 2013; Ciriello and Moreau, 2012) and inhibit the baroreceptor reflex (Arnold et al., 2009; Ciriello, 2013a, 2013b) at the level of the NTS. Interestingly, the NTS region has been identified as one of the few neurogenic sites within the central nervous system (Bauer et al 2005; Chigr et al 2009), and the NTS has a high level of expression of factors associated with neuroplasticity including the neurite outgrowth protein brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB), growth-associated protein-43 (GAP-43), and the synaptogenic marker synaptophysin (Jin et al., 2010; Korshunova and Mosevitsky, 2010; Moyse et al., 2006). Leptin has been shown to affect neuroplasticity and synaptogenesis within extra-hypothalamic regions of the brain (Harvey, 2013; Shanley et al., 2001; Wayner et al., 2004). Previous studies have suggested BDNF, TrkB, GAP-43 and/or synaptophysin may be important in mediating alterations associated with activity-dependent functional changes in cardiovascular afferents that terminate within NTS (Martin et al 2009).

This study was designed to investigate whether neuroplasticity occurred within NTS in response to acute (1 day), short term chronic (7 days) and long term chronic (95 days) IH and whether changes in mean arterial pressure (MAP) and heart rate (HR) occurred that may be associated with chronic IH. Finally, the role of leptin in the neuroplastic alterations in NTS following acute IH was also determined in the leptin deficient KILO rat (Vaira et al., 2012).

5.3 METHODS AND MATERIAL

5.3.1 Animals

Adult, male Sprague-Dawley rats (300-350 g; n=36) were purchased from Charles River Canada and served as wild-type control animals (WT). Adult, male, homozygous, leptin-deficient KILO rats (250-400 g; n=8; SD-$Lep^{tm1sage}$) were obtained from SAGE Laboratories (TGRA3780; Vaira et al., 2012). Animals were housed singly at a temperature of 22°C and 60% relative humidity with access to food and water available *ad libitum*, except during the daily 8 h IH or normoxic exposure, in 12h light/dark cycle conditions. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at The University of Western Ontario.

5.3.2 IH or normoxic exposure

Animals were exposed to 8 h (0900-1700) of IH or normoxia each day as previously described (Messenger et al., 2012, 2013; Moreau and Ciriello, 2013). WT rats were exposed for 1-day, 7-days or 95-days of IH or normoxia. KILO rats were exposed only for 1-day of IH or normoxia. In brief, animals were placed in a chamber consisting of four tubes (10 cm diameter x 35 cm length) with a zero-pressure escape valve. For IH-exposed animals, a computer that regulated solenoid valves altered the input of N_2 or room air to generate IH conditions. Animals were exposed to 80s hypoxia (6.5% O_2) followed by 120s normoxia. The levels of O_2 and CO_2 were monitored by sensors in the chamber, which relayed

information back to the computer to ensure proper cycling. Conditions within the chamber were isobaric (770 ± 11 mmHg) and eucapnic (<0.1% CO_2). Normoxic animals were exposed to same conditions except that only room air was cycled (Messenger et al., 2012, 2013; Moreau and Ciriello, 2013).

5.3.3 Hemodynamic recordings

Following exposure to IH or normoxia at 1 day, 7 days and 95 days, WT animals had their systolic, diastolic and MAP and HR measured using the non-invasive tail cuff method (CODA System; Kent Scientific; Torrington, CT). This approach has been previously validated with direct hemodynamic measures (Feng et al., 2008).

5.3.4 Tissue collection and preparation

Following exposure to IH or normoxia, animals were sacrificed under equithesin anesthesia (0.3 ml/100 g b.w.; Moreau and Ciriello, 2013) and the brains removed and frozen at -80°C until analyzed. Using a circular 1 mm (internal diameter) micropunch tool, 500 µm bilateral punches of NTS from each animal were taken and immediately homogenized in cold radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate) with protease inhibitor cocktail (Roche Applied Science; Laval, QC) using an electric homogenizer (VWR International; Radnor, PA). Homogenates were then sonicated over three passages for 15s on ice (55%;

Sonic Dimembrator Model 150; Fisher Scientific). Samples were then rotated for 10 min at 4 °C and centrifuged at 4°C for 20 min at 14000 RPM. Protein content of homogenates was quantified using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories; Hercules, CA). Protein samples were added to 25% LDS sample buffer and 10% reducing buffer (Life Technologies; Burlington, ON), and water to a standard protein concentration of 1.67 mg/ml (Messenger et al., 2013).

5.3.5 Western blots

Electrophoresis was carried out using a 10% discontinuous polyacrylamide Bis-Tris gel (Life Technologies; Burlington, ON), followed by standard protein immunoblotting techniques (Messenger et al., 2013). For each animal, 25 µg of protein of each sample was loaded. Electrophoresis was carried out at 200 V and terminated when the dye front reached the bottom of the gel. Proteins were transferred to a polyvinylidene fluoride membrane using a wet transfer method in the presence of methanol and SDS (50 mM Tris, 40 mM glycine, 0.3% SDS, 20% methanol) and wet transfer apparatus (Mini Trans-Blot Electrophoretic Transfer Cell; Bio- Rad Laboratories; Hercules, CA) at 100 V for 2 h. After transfer, the membrane was washed in Tris-buffered saline + Tween-20 (TBST; 20 mM Tris, 0.5 M NaCl, 0.1% Tween-20; pH 8.0) blocked for 1 h with 5% skim milk made in TBST buffer at room temperature. The membrane was then incubated with primary antibodies diluted in skim milk over night at 4 °C. The following day, the membrane was washed with TBST before being incubated with horseradish peroxidase-conjugated secondary antibodies-specific to the

appropriate host of the primary antibody being analyzed, for 1 h at room temperature. For detection, the membrane was washed with TBST, followed by distilled water and then detected using a horseradish peroxidase substrate ECL chemiluminescence system (Luminata Forte, EMD Millipore; Billerica, MA). Blots were visualized using a VersaDoc imaging system (Bio-Rad Laboratories; Hercules, CA) and analyzed using ImageLab v.3.0 (Bio-Rad Laboratories; Hercules, CA).

5.3.6 Antibodies

For western blots, the following antibodies were used: rabbit anti-β-actin-HRP (1:50000; A3854, Sigma-Aldrich; St. Louis MO), rabbit anti-TrkB (1:1000; sc-8316, Santa Cruz Biotechnology; Dallas, TX), mouse anti-synaptophysin (1:1000; ab8049, Abcam; Cambridge, MA), rabbit anti-GAP-43 (1:1000; #8945, Cell Signaling Technology; Danvers, MA), rabbit anti-BDNF (1:1000; sc-20981, Santa Cruz Biotechnology; Dallas, TX), donkey anti-rabbit IgG-HRP (1:10 000; 711-035-152, Jackson Immunoresearch; West Grove, PA), donkey anti-mouse IgG-HRP (1:5000; 715-035-151, Jackson Immunoresearch; West Grove, PA).

5.3.7 Statistics and analysis

All values are expressed as mean ± standard error. Differences between IH and normoxic groups were determined by unpaired, two-tailed Student t-test. A p-value < 0.05 was taken to indicate statistical significance. All bar charts were

made using GraphPad Prism v.5 graphing software (GraphPad Software; La Jolla, CA).

5.4 RESULTS

5.4.1 AP following exposure to IH

Following 1 day and 7 days of IH, systolic (Fig. 5.1a), diastolic (Fig. 5.1b), MAP (Fig. 5.1c), and HR (Fig. 5.1d) were found not to be altered compared to normoxic controls at the same time points. After 95 days of IH, animals had a significantly higher (p = 0.030) systolic blood pressure, compared to normoxic controls (152 ± 6 mmHg vs. 130 ± 8 mmHg, respectively; Fig. 5.1a). Additionally, these animals had significantly elevated diastolic blood pressure (p = 0.05; IH, 113 ± 6 mmHg vs. normoxic controls, 99 ± 4 mmHg; Fig 5.1b). As a result, MAP was significantly elevated within these animals (p = 0.026; IH, 124 ± 6 mmHg vs. normoxic controls, 107 ± 4 mmHg; Fig 5.1c). On the other hand, no differences (p = 0.13) were observed in HR between the normoxic control and IH groups (400 ± 12 bpm vs. 419 ± 12 bpm, respectively; Fig. 5.1d). Figure 5.1e also shows that the gain as measured by the MAP level compared to HR was decreased at 95 days following IH compared to normoxic controls.

5.4.2 BDNF protein expression in NTS following IH

BDNF protein expression was found not to be different (p = 0.42) in NTS between IH and normoxic WT after 1 day exposure (Fig. 5.2a). Following 7 days of IH exposure, there was 53% increase (p = 0.0062) in BDNF protein expression in NTS compared to normoxic WT (Fig 5.2b). On the other hand, after 95 d of IH exposure, there was 34% less BDNF protein expression in NTS compared to normoxic WT (p = 0.008; Fig 5.2c).

Figure 5.1 Effect of IH on hemodynamic variables

Bar chart showing arterial pressure and heart rate changes following 1, 7 and 95 days of exposure to IH or normoxia. Shown are: (a), systolic pressure, (b), diastolic, (c) mean arterial pressure and (d) heart rate in the conscious rat. In addition, (e) shows the effect of IH on heart rate in relation to the level resting arterial pressure (Gain; bpm/mmHg). Note that systolic, diastolic and mean blood pressures and gain are significantly (*, $p<0.05$) altered after 95 days of IH exposure. n=7 for all the 1 and 7 days groups and n=8 for the 95 day groups.
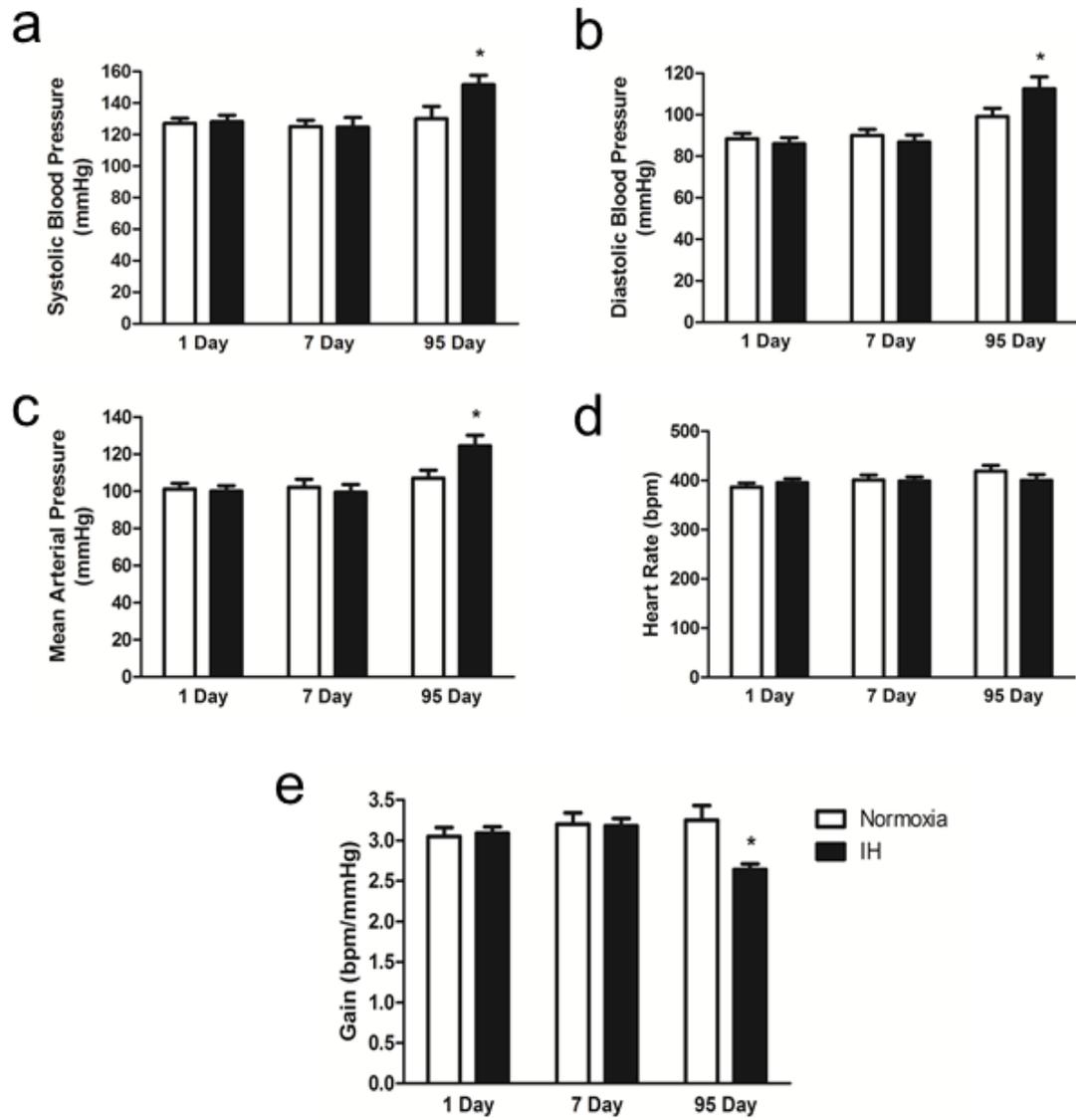
Figure 5.2 NTS expression of BDNF following IH

Representative western blots and bar charts indicating the change in protein expression of BDNF determined by western blot in the same NTS samples at 1 day (a), 7 days (b) and 95 days (c) after IH or normoxic exposure. Note that BDNF protein expression is elevated after 7 days of IH exposure and then reduced after 95 days of IH exposure. *, p<0.05 determined by two-tailed, unpaired Student t-test. n=7-8.

a 1 Day

BDNF

β-Actin

BDNF/β-Actin Protein (A.U.)

Normoxia   IH

b 7 Days

BDNF

β-Actin

BDNF/β-Actin Protein (A.U.)

Normoxia   IH

c 95 Days

BDNF

β-Actin

BDNF/β-Actin Protein (A.U.)

Normoxia   IH
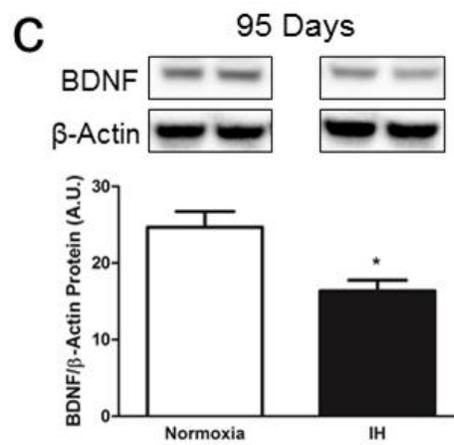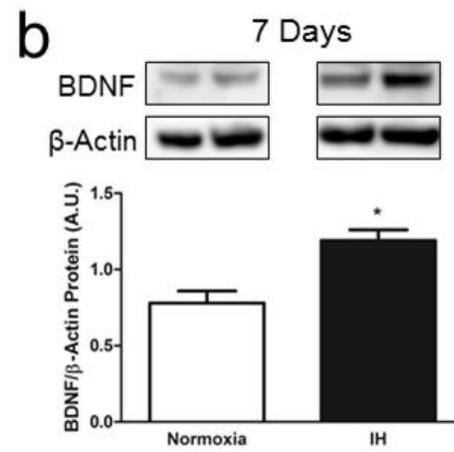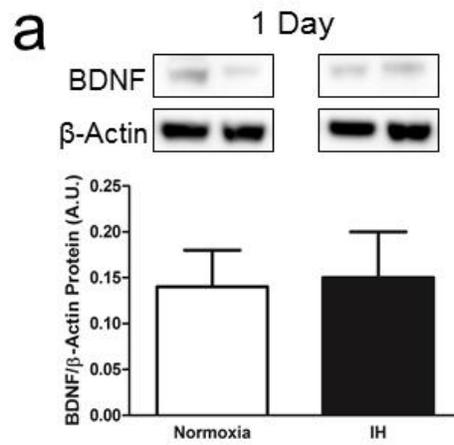
5.4.3 TrkB protein expression in NTS following IH

After 1 day of IH, there was a significant elevation in protein expression of both the short (gp95) and long (gp145) isoforms of the TrkB neurotropic receptor in the NTS region of WT (Fig 5.3a). The gp95 isoform was increased ($p = 0.028$) by about 28% in IH compared to normoxic controls (Fig. 5.3). Over the same time period in the NTS, the gp145 isoform was also increased ($p = 0.018$) by about 166% in IH compared to normoxic controls (Fig. 5.3a). The ratio of gp145:gp95 isoform was 100% higher ($p = 0.012$) in IH compared to normoxic control NTS in WT (Fig. 5.3d).

Following 7 days of IH (Fig 5.3b), gp95 was significantly higher ($p = 0.043$) from normoxic controls in NTS, with about 43% more gp95 protein expression in IH animals. The gp145 isoform was also significantly greater ($p = 0.038$) by about 52% in IH compared to normoxic controls (Fig. 5.3b). The ratio of gp145:gp95 isoform was not significantly different ($p = 0.39$) in IH compared to normoxic control (Fig. 5.3d).

After 95 days of IH (Fig 5.3c), gp95 was significantly lower ($p = 0.013$) by approximately 52% compared to normoxic control animals, while the gp145 isoform was not different ($p = 0.27$). The gp145:gp95 ratio was also not different ($p = 0.29$) between 95 d IH exposed and 95 d normoxic animals (Fig. 5.3d).

5.4.4 Synaptophysin protein expression in NTS following IH

The expression of the synaptic marker protein synaptophysin was significantly lower ($p = 0.042$) by about 16% in NTS after 1 day IH exposure compared to

Figure 5.3 NTS expression of TrkB isoforms following IH

Representative western blots and bar charts indicating the change in protein expression of gp95 (a), gp145 (b) and the ratio of gp145:95 (c) TrkB isoforms in NTS following 1 day (a), 7 days (b), and 95 days (c) IH or normoxic exposure. (d), the ratio of gp145:gp95 TrkB isoforms. Note that both TrkB isoforms are increased after acute and short term exposure to IH. However, after 95 days IH exposure, only the gp95 isoform is reduced. Differences were determined between IH and normoxia within isoform and ratio of each group. *, $p < 0.05$ as determined by two-tailed, unpaired Student t-test. n=7-8.
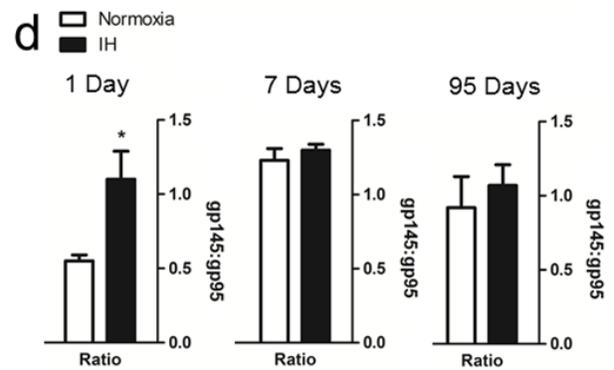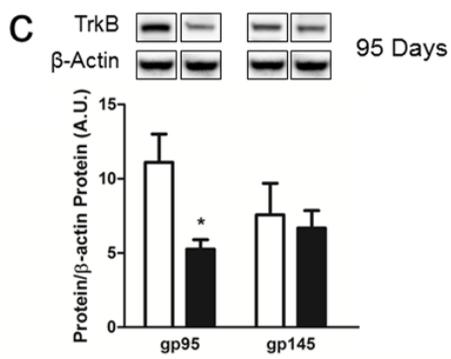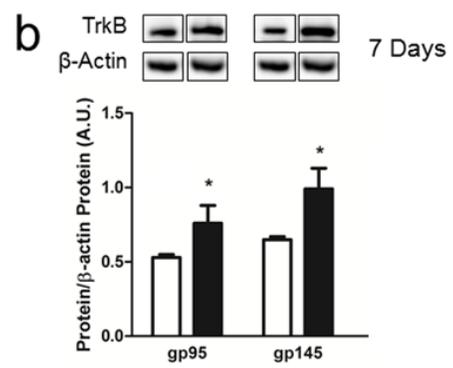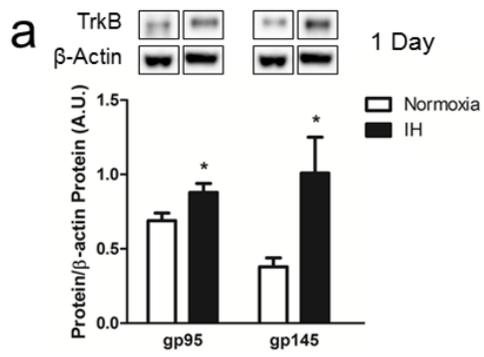
normoxic control WT (Fig 5.4a). On the other hand, no differences were found in synaptophysin protein expression (p = 0.34) between IH and normoxic controls in the NTS region following 7 days of exposure (Fig 5.4b), although there was a trend towards a decrease. After 95 days, there was about 42% less expression (p = 0.0029) of the synaptophysin protein in NTS of the IH animals compared to normoxic WT animals (Fig 5.4c).

5.4.5 GAP-43 protein expression in NTS following IH

GAP-43 protein was not different in NTS between IH and normoxic WT rats at 1 day (p = 0.42) and at 7 days (p = 0.48) exposure (Fig 5.5a-b). Following 95 days, GAP-43 protein expression was reduced by about 34% (p = 0.045) in NTS of the IH exposed animals compared to the normoxic animals (Fig 5.5c).

5.4.6 Changes in neuroplastic markers in NTS of KILO rats following IH

To determine whether leptin contributed to the changes observed in the neuroplastic markers in NTS, leptin deficient rats (KILO rat) were exposed to acute (1 day) IH. IH failed to induce any changes in BDNF in the KILO (p=0.44) or WT rat (Fig. 5.6a). Similarly, in the KILO rat, acute IH did not induce changes in TrkB isoforms (Fig. 5.6b) which were present in the WT rat. Neither gp95 (p = 0.27; Fig. 5.3b), gp145 (p = 0.20) nor the ratio of gp145:gp95 TrkB isoforms (p = 0.44) were altered in NTS of the KILO rats following IH exposure. On the other hand, 1 day exposure of KILO rats to IH induced a lower (p = 0.043) protein expression of synaptophysin in NTS by about 26% compared to normoxic KILO

Figure 5.4 NTS expression of synaptophysin following IH.

Representative western blots and bar charts showing changes in protein expression of synaptophysin in NTS after 1 day (a), 7 days (b) and 95 days (c) of exposure to IH or normoxic. Note the synaptophysin decreases in response to acute/short term and long term IH exposure. *, $p<0.05$ as determined by two-tailed, unpaired Student t-test. n=7-8.
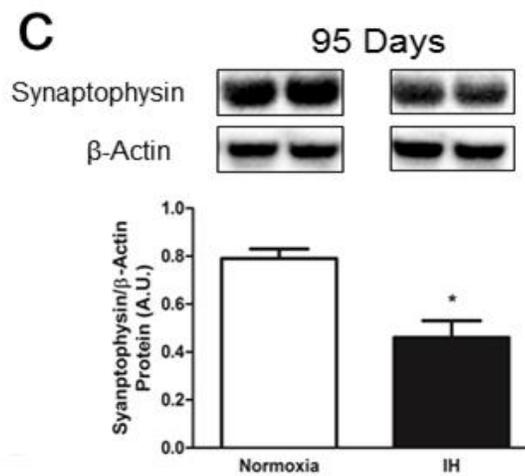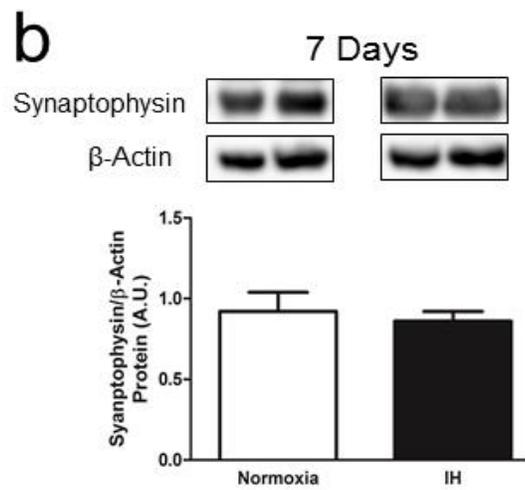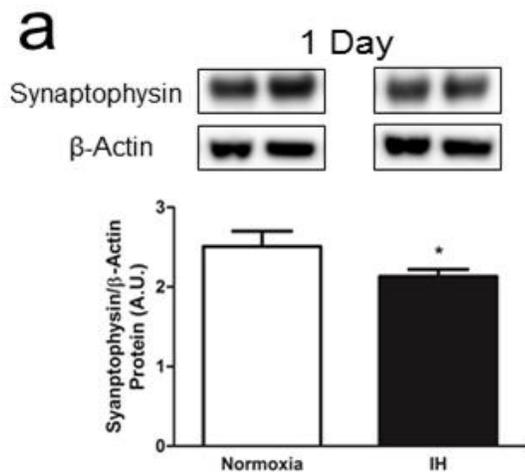
Figure 5.5 NTS expression of GAP-43 following IH.

Representative western blots and bar charts showing the change in protein expression of GAP-43 in NTS at 1 day (a), 7days (b) and 95 days (c) after IH or normoxic exposure. Note that at only 95 days of IH exposure is GAP-43 altered in NTS. *, $p < 0.05$ as determined by two-tailed, unpaired Student t-test. n=7-8.

a 1 Day

b 7 Days

c 95 Days
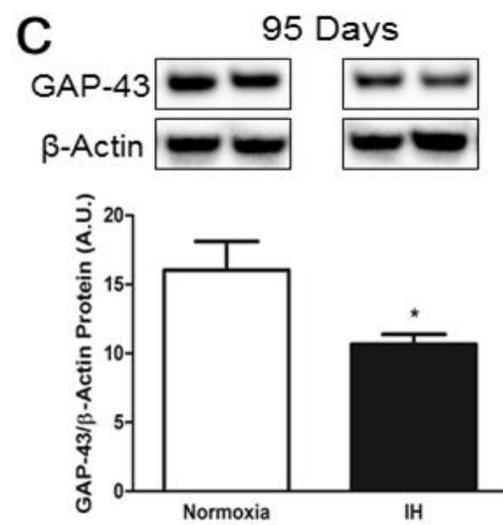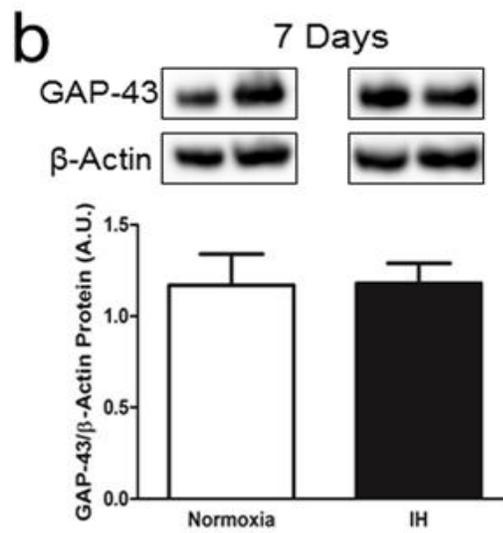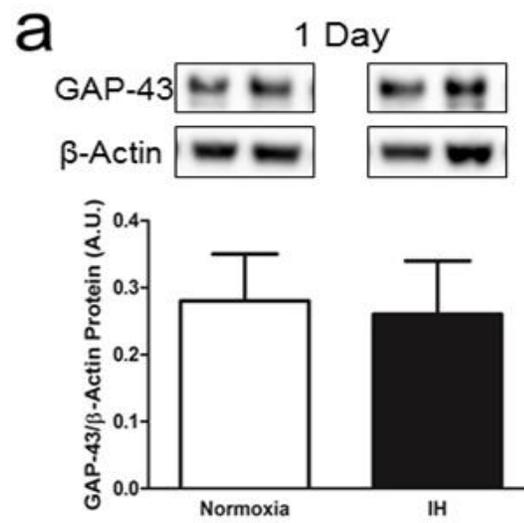
controls (Fig. 5.6c). IH as previously shown induced a decrease in synaptophysin protein expression in the WT rat after 1 day exposure. Acute IH exposure in the KILO rat did not induce changes in GAP-43 (p = 0.44; Fig. 5.6d).

Figure 5.6 Effect of IH in KILO rats on neuroplastic markers in NTS.

Representative western blots and bar charts showing the change in protein expression of BDNF (a), TrkB (b), synaptophysin (c)m and GAP-43 in NTS at 1 day IH exposure in WT and KILO$^{-/-}$ rats. Note that the lack of leptin abolishes the TrkB effects to IH (b), but not those associated with synaptophysin (c) after IH. In addition, note that the lack of leptin does not induce changes in BDNF nor GAP-43 after IH. *, $p<0.05$ as determined by two-tailed, unpaired Student t-test. n=4-8.

a

BDNF

β-Actin

1 Day

BDNF/β-Actin Protein (A.U.)

Normoxia
IH

W/T   KILO⁻/⁻

b

TrkB gp95

β-Actin

TrkB gp95/β-actin Protein (A.U.)

W/T   KILO⁻/⁻

c

Synaptophysin

β-Actin

Syanptophysin/β-Actin Protein (A.U.)

W/T   KILO⁻/⁻

d

GAP-43

β-Actin

GAP-43/β-Actin Protein (A.U.)

W/T   KILO⁻/⁻
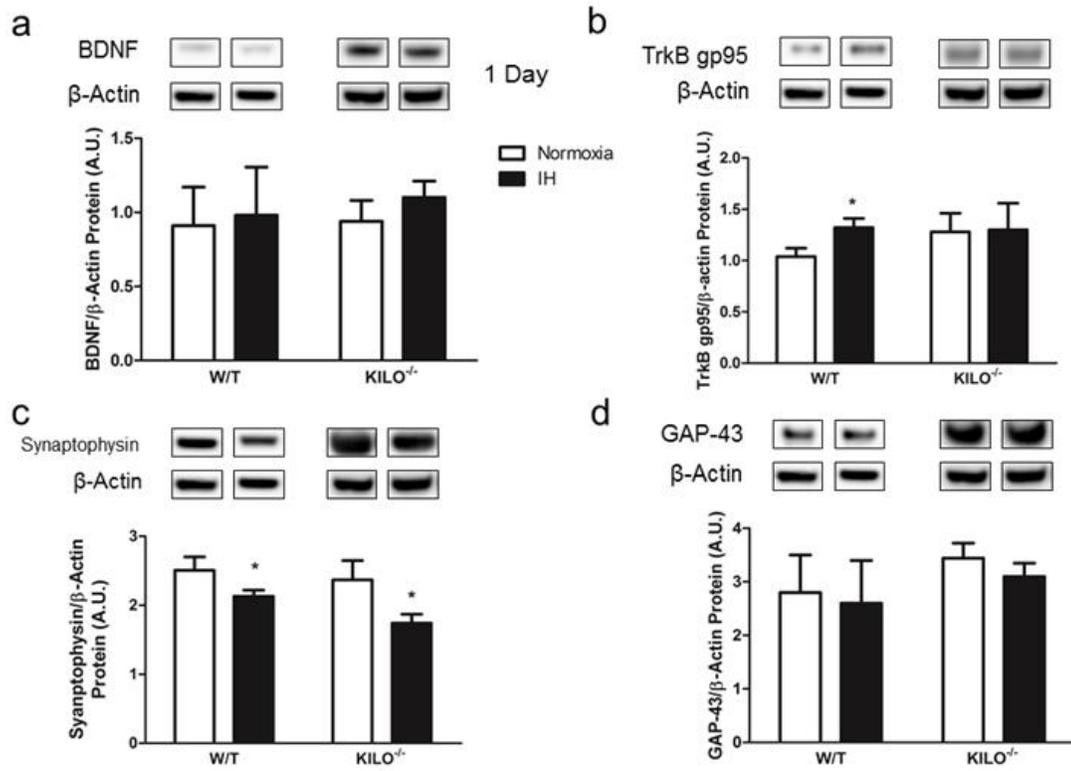
5.5 DISCUSSION

This study has demonstrated that both acute and long term IH exposure induces changes in neuroplasticity markers in NTS. In addition, some of these changes observed following acute IH exposure were due to leptin signaling in NTS as they were not observed in the leptin deficient rats. Finally, long term exposure to IH induced an increase in MAP, but not HR, suggesting that some of the neuronal plasticity changes observed in NTS may be associated with the hypertension resulting from long term activation of peripheral chemoreceptors.

BDNF protein expression levels were elevated in NTS following 7 days of IH exposure. On the other hand, after 95 days of IH exposure, BDNF levels were found to be lower than those of animals exposed to normoxia. These changes suggest an initial elevated responsiveness of the BDNF-TrkB system, whereas this effect is diminished over long-term exposure to IH as gp145:gp95 normalizes, and BDNF ligand is reduced. The neurotrophin BDNF has been demonstrated in NTS cardiovascular afferent synapses (Martin et al., 2009), and has been suggested to be released in response to activation of the carotid chemoreceptor reflex (Chavez-Valdez et al., 2012; Montero et al., 2012). BDNF-containing terminal boutons come in close apposition to second-order neurons shown to express its receptor, TrkB (Kline et al., 2010). Within NTS, BDNF has been reported to modulate glutamatergic neurotransmission and thus altering cardiovascular responses (Clark et al., 2011).

It was found that the TrkB receptor isoforms within NTS increased in response to acute and short term IH exposure, whereas after 95 day IH

exposure, the gp95 isoform was decreased. The gp145 TrkB subunit is considered the full-length isoform of the receptor, whereas gp95 TrkB is a truncated version lacking an intracellular kinase domain (Klein et al., 1990; Middlemas et al., 1991). The gp95 TrkB acts as a dominant negative receptor inhibiting the responsiveness of the full-length gp145 TrkB to stimulation by BDNF (Haapasalo et al., 2001). Due to the dominant negative nature of the gp95 TrkB isoform, the ratio of gp145:gp95 may be an important indicator of BDNF-TrkB signaling capability (Haapasalo et al., 2001). This ratio was found to be higher in animals exposed to 1 day IH, but not different following 7 or 95 days of exposure. As BDNF-TrkB signaling plays an important role in long-term neuroplastic alterations such as neurite outgrowth (Hartnick et al., 1996; Hu et al., 2005) and synaptogenesis (Seil and Drake-Baumann, 2000; Hu et al., 2005), it is likely that these changes begin within the first day of IH exposure and diminish over time once neuroplasticity has been induced. The finding that BDNF protein expression decreases after long term exposure is consistent with a recent study by Chavez-Valdez and colleagues (2012) demonstrating a decrease of NTS BDNF content following hyperoxia in juvenile rats.

TrkB may also mediate the effects of hormonal signaling molecules such as leptin within NTS (Spaeth et al., 2012) and other brain regions (Liao et al., 2012). The changes in TrkB isoforms observed following acute IH were not seen in NTS of leptin-deficient KILO rats. This observation, coupled with the earlier finding that plasma leptin levels are elevated after acute IH (Messenger et al., 2012; Moreau and Ciriello, 2013) suggests that leptin is important for the

increase in TrkB. Although the importance of leptin over longer periods of IH exposure is not known and warrants further study, on the basis of the finding that TrkB protein expression remains elevated compared to normoxic controls after short term IH exposure suggests that it may continue to signal TrkB. Interestingly, the decrease in TrkB after 95 days of IH exposure is also consistent with the finding of decreased plasma levels of leptin observed in these animals after long term IH exposure (unpublished observations).

We observed reduced synaptophysin during exposure to IH and reduced GAP-43 protein expression only following 95 days of IH. The reduction in synaptophysin in NTS following 1 day exposure of IH was not due to leptin signaling as it was still observed within the leptin deficient animals. This suggests that these alterations in synaptophysin expression may be due to activity-dependent signaling (Li et al., 2002) or a direct effect of hypoxia (Ding et al., 2009). Synaptophysin is a marker for synapses and synaptic strength, and has been used for the quantification of synapses (Calhoun et al., 1996). On the other hand, GAP-43 is an important mediator of neurite outgrowth (Jap Tjoen San et al., 1991), neuronal regeneration (Meiri et al., 1988) and a marker of the pre-synaptic terminals (Eastwood et al., 2007). Consistent with the findings in this study, it has recently been shown that IH exposure reduces afferent neurotransmission in the intermediate and caudal NTS (Almado et al., 2012). It was also determined that this synaptic depression was the result of a loss of active synapses (Almado et al., 2012). Thus, the reduction in synaptophysin and GAP-43 observed in this study could be interpreted to suggest a reduction in

NTS active synapses, synaptic strength and afferent neurites. Although it would be expected that chemoreceptor afferent activity would be elevated following IH, the depression of neurotransmission may be selectively related to reduced baroreceptor reflex activity and output observed in animals exposed to chronic IH (Lai et al., 2006). Consistent with this suggestion, it was found that after chronic IH, the animals exhibited an elevated MAP and a decreased HR response to the elevated arterial pressure further suggesting an impairment of the baroreceptor reflex.

In conclusion, the present results support the notion that the NTS complex functions as an important site of integration of afferent inputs. The IH induced changes observed in neuroplastic molecules including BDNF-TrkB, synaptophysin and GAP-43 suggest this integration is dynamic, and may be altered over acute, short- and long-term IH and at least the short term effects appear to be dependent on leptin. The direct contribution of these alterations to cardiovascular changes observed are not known, but do appear to be associated with hypertension and possibly baroreceptor reflex resetting.

5.6 FOOTNOTES

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CHAPTER 6

EFFECTS OF ANGIOTENSIN II ON LEPTIN AND DOWNSTREAM LEPTIN

SIGNALING IN THE CAROTID BODY DURING ACUTE INTERMITTENT

HYPOXIA

6. CHAPTER 6

6.1 CHAPTER SUMMARY

Angiotensin II (ANG II) is known to promote leptin production and secretion. Although ANG II type 1 receptors (AT_1R) and leptin are expressed within the carotid body, it is not known if AT_1R and leptin are co-expressed in the same glomus cells nor if these peptides interact to alter the carotid body function. This study was done to investigate whether ANG II altered leptin signaling in the carotid body during acute intermittent hypoxia (IH). Rats were treated with captopril or the AT_1R blocker losartan in the drinking water for 3 days prior to being exposed to IH (8h) or normoxia (8h). IH induced increases in plasma ANG II and leptin compared to normoxic controls. Captopril treatment abolished the plasma leptin changes to IH, whereas losartan treatment had no effect on the IH induced increase in plasma leptin. Additionally, carotid body glomus cells containing both leptin and the long form of the leptin receptor (OB-Rb) were found to co-express AT_1R protein, and IH increased the expression of only AT_1R protein within the carotid body in both captopril and non-captopril treated animals. On the other hand, losartan treatment did not alter AT_1R protein expression to IH. Additionally, captopril and losartan treatment eliminated the elevated carotid body leptin protein expression, and the changes in phosphorylated signal transducer and activator of transcription 3 protein, the short form of the leptin receptor ($OB-R_{100}$), suppressor of cytokine signaling 3, and phosphorylated extracellular-signal-regulated kinase 1/2 protein expression induced by IH. However, captopril elevated the expression of OB-Rb protein,

whereas losartan abolished the changes in OB-Rb protein to IH. These findings, taken together with the previous observation that ANG II alters carotid body chemosensitivity, suggest that the increased circulating levels of ANG II and leptin induced by IH act at the carotid body to alter leptin signaling within the carotid body which in turn may influence chemoreceptor function.

6.2 INTRODUCTION

The carotid body is a highly vascularized organ located bilaterally at the bifurcations of the common carotid arteries (Heymans et al., 1930; Nurse, 2005). The carotid body, composed of specialized oxygen-sensing type-I glomus cells, signals carotid sinus nerve endings that transmit the afferent information to the nucleus of the solitary tract (Ciriello et al., 1994), activating homeostatic mechanisms involving respiratory, cardiovascular and hormonal systems (Schultz and Li, 2007; Kumar, 2009).

There are now considerable data suggesting that substances within the circulation gain access to carotid body glomus cells and alter their excitability (Allen 1998; Chen et al., 2005; Leung et al., 2000, 2003; Nurse & Piskuric, 2012; Peng et al., 2006). One vasoactive compound shown to alter the discharge of carotid body afferent fibers independent of its circulatory effects is angiotensin II (ANG II) (Allen 1998; Leung et al., 2000). The carotid body has been shown not only to contain ANG II binding sites (Allen, 1998), but to also possess its own intrinsic renin-angiotensin system (RAS) (Lam & Leung, 2002), suggesting that ANG II within the carotid bodies may act in an autocrine/paracrine manner. Key elements of the RAS, including protein and mRNA of angiotensinogen as well as mRNA of angiotensin I converting enzyme (ACE) have been localized to the type-I glomus cells (Lam & Leung, 2002). A role for ANG II in chemosensitivity is supported by the finding that gene expression for the ANG II type 1 receptor (AT_1R) is up-regulated in the carotid bodies during chronic hypoxia (Leung et al., 2000; Fung et al., 2002), and this increased expression of the AT_1R is associated

with the increased sensitivity of carotid chemoreceptors (Leung et al., 2000; Fung et al., 2001, 2002). In addition, administration of the ANG II type 1 receptor (AT_1R) antagonist losartan abolishes these effects on chemoreceptors (Allen, 1998; Leung et al., 2000), including the increased intracellular calcium levels in type-I cells induced by ANG II (Fung et al., 2001). Consistent with these results, a recent study has demonstrated that signaling through the AT_1R is critical for carotid chemoreceptor sensitivity and signal transduction of the carotid chemoreceptor reflex during hypoxia (Marcus et al., 2010).

The increases in intracellular calcium induced by ANG II suggest that through activation of the AT_1R, ANG II may impact vesicle secretion from type-I glomus cells and therefore promote the release of other potential modulators involved in chemoreceptor function and for the signal transduction of glomus cells to the carotid sinus nerve (Gomez-Nino et al., 1990; Eyzaguirre and Zapata, 1968; Bock, 1980). One possibility may be that ANG II may signal changes in leptin signaling within the carotid body as ANG II signaling is known to affect leptin production and secretion in other tissues such as ventricular myocytes, bone marrow and adipocytes (Cassis et al., 2004; Danser et al., 1999; Haznedaroglu and Buyukasik, 1997; Haznedaroglu et al., 1996; Rajapurohitam et al., 2006, 2012). Leptin is a 16 kDa protein product of the *obese* gene and is produced in proportion to, and secreted primarily, but not exclusively, by adipocytes (Lonnquist et al., 1995; Caro et al., 1996). Circulating leptin is thus an indicator of body fat content and acts as a satiety hormone, as well as increasing energy expenditure to balance body energy stores (Lonnquist et al., 1995; Caro

et al., 1996). Leptin signals primarily through the long-form leptin receptor (OB-Rb), although 6 splice variants of the leptin receptor exist (OB-Ra-OB-Rf) of which only a few have known functions (Lee et al., 1996; Wang et al., 1996). Leptin has been shown to possess a signaling capability within the carotid body (Messenger et al., 2012; Messenger and Ciriello, 2013), and both circulating and carotid body protein expression levels of leptin have been found to increase in response to acute intermittent hypoxia (IH) and chronic intermittent hypoxia challenge (Messenger et al., 2012; Messenger and Ciriello, 2013), although its function within carotid body glomus cells remains unclear. The discovery of leptin, as well as four leptin receptor isoforms within carotid body glomus cells (Messenger et al., 2012, 2013; Porzionato et al., 2011), as well as the finding that circulating leptin induces phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and immediate early gene Fra-1/2 expression within glomus cells (Messenger et al., 2012) is suggestive of a role in chemosensitivity for the adipokine.

ANG II has been shown to be released and is required along with increased sympathetic nerve activity for the chronic hypertension in both human and the animal model of obstructive sleep apnea (Fletcher et al., 1999, 1992a/b; Moller et al., 2003; Yuan et al., 2004). In addition, in the animal model of obstructive sleep apnea, circulating and carotid body leptin is elevated (Messenger et al., 2012; Messenger and Ciriello, 2013; Moreau and Ciriello, 2013), a finding consistent with clinical data (Phillips et al., 2000; Harsch et al., 2003). However, it is not known whether ANG II may affect leptin signaling in the

carotid body, which in turn may contribute to altered sensitivity within the chemoreceptor reflex pathway (Ciriello and Moreau, 2012, 2013). Therefore, this study was done to determine: (1) whether, selective chronic inhibition of ACE by captopril or the selective blockade of the AT_1R by losartan had an effect on circulating levels of leptin during IH; (2) whether glomus cells expressing the AT_1R co-express leptin and the OB-Rb, and whether AT_1R levels in carotid bodies are altered following exposure to IH; (3) whether chronic inhibition of ACE or the AT_1R alters leptin, leptin receptor or downstream mediators of the OB-Rb signaling within the carotid body.

6.3 METHODS AND MATERIAL

6.3.1 General animal procedures

Experiments were done in male Sprague–Dawley rats (Charles River Canada, St. Constant, Canada) weighing 250-350 g. All animals were housed under controlled conditions with a 12 h light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

6.3.2 Angiotensin converting enzyme inhibition

Inhibition of angiotensin converting enzyme was performed by administration of captopril (Sigma-Aldrich, St. Louis, MO) in the drinking water (2 mg/ml) of each animal three days prior to IH (n=7) or normoxic conditions (n=7). Captopril was freshly dissolved daily in the drinking water which was monitored daily and it was calculated that the animals received 146 ± 6 mg/kg/day of captopril. At this dose, ACE inhibition has been shown to occur (Schiffrin & Genest, 1982; Ferrone & Antonaccio, 1979).

6.3.3 Selective blockade of AT_1R

Selective blockade of the AT_1R was accomplished by the administration of losartan (Merck, Whitehouse Station, NJ) in the drinking water (0.12 mg/ml) of each animal three days prior to AIH (n=7) or normoxic conditions (n=7). Losartan was freshly dissolved daily in the drinking water which was monitored daily and it was calculated that the animals received 14.2 ± 0.7 mg/kg/day of losartan. At this dose, AT_1R blockade has been shown to occur (Boustany et al., 2005; Kline & Liu, 1994).

6.3.4 Induction of IH

IH or normoxia was induced in the captopril or losartan treated animals described above and in a separate set of animals that were not treated with the drugs (IH, n=11; normoxia, n=11) as previously described (Messenger et al., 2012; Messenger and Ciriello, 2013; Moreau and Ciriello, 2013). Animals were exposed to the 8h IH or the normoxic stimuli each day of the experimental period during daylight hours (900h-1700h).

6.3.5 Plasma collection and immunoassays

Measurement of plasma levels of leptin and ANG II were made immediately after the exposure of the animals to IH or the normoxic stimuli (Messenger et al.,

2012; Messenger & Ciriello 2013; Moreau and Ciriello, 2013). Blood samples were collected by cardiac puncture in 7% ethylenediaminetetraacetic acid at a volume of 10 μl/ml blood. The blood was immediately centrifuged at 10 000 RPM for 10 min at 4 °C to isolate the aqueous plasma. This aqueous plasma phase was removed and stored frozen at -80 °C until analyzed for hormone content. Plasma samples were analyzed using enzyme immunoassays for rat leptin (inter-assay variability: 6.5% intra-assay variability: 7.1%; Enzo Life Sciences; Farmingdale, NY), and rat ANG II (inter-assay variability: <15%; intra-assay variability: <10%; Phoenix Pharmaceuticals, Burlingame, CA) according to manufacturer instructions. Enzyme immunoassay plates were read on a SpectraMax M5 plate reader using SoftMax Pro v.5 microplate analysis software (Molecular Devices; Sunnyvale, CA).

6.3.6 Carotid body protein extracts and immunoblot analysis

Carotid body protein extracts were obtained following bilateral carotid body excision from the normoxia- or IH-exposed animals as described previously (Messenger et al., 2012; Messenger & Ciriello 2013). In brief, the carotid bodies (bilaterally) from each animal were snap frozen, pooled together, homogenized in 200μl of RIPA buffer solution (150 mM NaCl, 1 mM NaF, 1 mM NaVO$_4$, 0.5 mM β-glycerophosphate, 1mM EDTA, 1% Triton-X 100, 50mM Tris-HCL at pH of 7.5) with a protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada) and sonicated. The homogenate was centrifuged at 4°C for 15 min at 2100 RPM. The

supernatant was retained and then centrifuged a second time at 4°C for 20 min at 13200 RPM. The resultant supernatant was retained as the protein preparation. Equal concentrations of extracted proteins normalized by colorimetric DC assay (Bio-Rad laboratories, Hercules, CA) underwent gel electrophoresis in 10% Bis-acrylamide gel (Life Technologies Inc., Carlsbad, CA) and transferred onto a polyvinylidene fluoride membrane. Blots were probed using: polyclonal rabbit anti-AT_1R (1:1000; Cat. # sc-1173; Santa Cruz Biotechnology Inc., Dallas, TX), anti-AT_2R (1:1000; Cat. # sc-7420; Santa Cruz), polyclonal rabbit anti-leptin (1:1000, Cat. # sc843; Santa Cruz Biotechnology Inc.), polyclonal rabbit anti-OB-R (1:1000, Cat. # OBR12-A; Alpha Diagnostics International Inc., San Antonio, TX), affinity purified polyclonal chicken anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics, Edina, MN), polyclonal rabbit anti-SOCS3 (1:1000, Cat. # ab16030; Abcam Inc., Cambridge, MA), monoclonal rabbit anti-STAT3 (1:2000, Cat. # 4904S; Cell Signaling Technology), polyclonal rabbit anti-pSTAT3 (1:1000, Cat. #9131S; Cell Signaling Technology, Danvers, MA), monoclonal rabbit anti-ERK1/2 (1:1000, Cat. # 4695S; Cell Signaling Technology), monoclonal rabbit anti-pERK1/2 (1:1000, Cat. # 4376S; Cell Signaling Technology), and monoclonal horseradish peroxidase-conjugated β-actin (1:50000, catalog no. A3854, Sigma-Aldrich) diluted in 5% milk-Tris-buffered saline-Tween 20 buffer and with horseradish peroxidase conjugated donkey anti-rabbit IgG, donkey anti-goat, or donkey anti-chicken IgG (1:10000, catalog # 711- 035-152, 705-035-003 or 703-035-155, respectively; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 5% milk-1X Tris-

buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Millipore Canada Ltd, Toronto, ON, Canada).

6.3.7 Immunofluorescence

Immediately after the application of the 8h IH or normoxic stimuli, the animals (IH, n=3; normoxia, n=3) were anesthetized with 0.3 ml/100g equithesin and perfused transcardially using 500 ml ice-cold phosphate buffered saline (PBS; 0.1M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (Messenger et al., 2012; Messenger & Ciriello 2013). The carotid arteries at their bifurcations, along with the attached carotid bodies were removed bilaterally, and stored overnight in Zamboni's fixative at 4 °C. The following day the tissues were gradually dehydrated through a series of alcohols, and placed in xylene followed by paraffin wax. Serial transverse sections were cut at 6 µm on a RM 2155 microtome (Leica Microsystems Inc., Buffalo Grove, IL), floated in a warm water bath and mounted on double-gelatinized glass microscope slides and placed on a slide warmer. Tissue sections were later de-paraffinized in xylene and rehydrated using graded alcohol solutions. For each animal, 1 in every 5 slides was stained with thionin to identify the region of the carotid body and blood vessels. Additionally, adjacent sections to those stained for thionin from the normoxic and IH-exposed animals were rinsed in PBS and processed for double-immunofluorescence (Vectastain Elite ABC Kit, Cat. # PK6100; Vector

Laboratories, Burlingame, CA) for leptin, Ob-Rb and AT_1R. Sections underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate/0.05% Tween; pH 6.0) heated to 90-95ºC in a microwave for 15 min and then were rinsed in PBS before being placed in 5% normal goat serum ((Cat. # S-1000; Vector Laboratories) in PBS containing 0.3% Triton X-100 for 30 min (Messenger et al., 2012). The sections were later rinsed in PBS and placed overnight (12 h) into a primary polyclonal rabbit anti-leptin (Cat. # sc843; Santa Cruz Biotechnology Inc.; 1:1000 in PBS/0.3% Triton X-100) or affinity purified polyclonal chicken anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics) at room temperature. Sections were then rinsed in PBS and incubated in goat biotinylated anti-rabbit IgG (Cat. # BA-1000; Vector Laboratory) diluted 1:500 in PBS/ 0.3% Triton X-100 for 1 h. Following PBS rinses, sections were placed in Streptavidin Alexafluor-488 (Cat. # S11223; Life Technologies) diluted 1:100 for 1 h. Following PBS rinses, sections were exposed to an avidin containing solution for 15 min followed by a biotin containing solution for an additional 15 min (Avidin/Biotin blocking kit, Cat. # SP-2001; Vector Laboratories) to block non-specific binding sites. Sections were then rinsed in PBS and incubated overnight at room temperature in primary polyclonal rabbit anti-AT_1R (Cat. # sc-1173; Santa Cruz Biotechnology Inc.) diluted 1:1000 in PBS/0.3% Triton-X 100 and 5% normal goat serum. Following PBS washes, the sections were placed in goat biotinylated anti-rabbit IgG diluted 1:500 in PBS/ 0.3% Triton X-100 or goat biotinylated anti-chicken IgY (Vectastain Elite ABC Kit) diluted 1:200 in PBS/ 0.3% Triton X-100 for 1 h. Sections were rinsed in PBS and placed in

Streptavidin Texas Red (Code: RPN1233; GE Healthcare, Baie d'Urfe, QC, Canada) diluted 1:100 for 1 h. Following PBS rinses, sections were placed in 100% ethanol containing 5% glacial acetic acid on dry ice for 10 min. Sections were then immediately cover-glassed using Fluoromount mountant.

6.3.8 Statistical analysis

Statistical comparisons between the normoxia and IH exposed animals were made using a two-tailed, unpaired Student t-test. In all comparisons, a minimum p-value of < 0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

6.4 RESULTS

6.4.1 Plasma ANG II and leptin levels following captopril or losartan treatment

IH significantly increased plasma ANG II ($p < 0.002$) (Fig. 6.1a) levels about 3 fold above those found in the normoxic controls. Plasma leptin (Fig. 6.1b) levels were also significantly increased in IH exposed animals by approximately 6 fold ($p < 0.01$) above those found in the normoxic controls. Following captopril treatment, no differences were found between the IH and normoxic controls in both ANG II and leptin levels within the plasma. Following losartan treatment ANG II levels were found not to be different between the normoxic controls and IH exposed animals (Fig. 6.1a), whereas the increased levels of leptin induced by IH was not affected by the selective blockade of AT_1R with losartan (Fig. 6.1b).

6.4.2 Leptin, OB-Rb and AT_1R co-expression in carotid body

Figure 6.2 shows the effect of IH on leptin, OB-Rb and AT_1R immunoreactivity within glomus cells of the carotid body. Glomus cells expressed low levels of leptin (Fig. 6.2a), OB-Rb and AT_1R (Fig. 6.2b) immunofluorescence under normoxic conditions. However, following exposure to IH, carotid body glomus cells exhibited an elevated immunofluorescence associated with leptin (Fig. 6.2c), ObRB (Fig. 6.2e), and AT_1R (Fig. 6.2d and 6.2f). Figure 6.2 also shows

Figure 6.1 Change in plasma levels of ANG II and leptin following IH.

Bar charts showing the effect of IH on plasma levels of ANG II (a), and leptin (b) compared to levels observed in normoxic controls under sham (non-captopril treatment), and captopril and losartan treatment. Note that plasma ANG II (a) and leptin (b) are significantly elevated following IH in the non-captopril treated (sham) group compared to normoxic group. Additionally, note that captopril and losartan prevented the rise in ANG II to IH. Furthermore, note that while captopril blocked the rise in leptin to IH, this increase in leptin to IH was not affected by losartan treatment. Values are shown as means ± S.E. *$p < 0.01$.
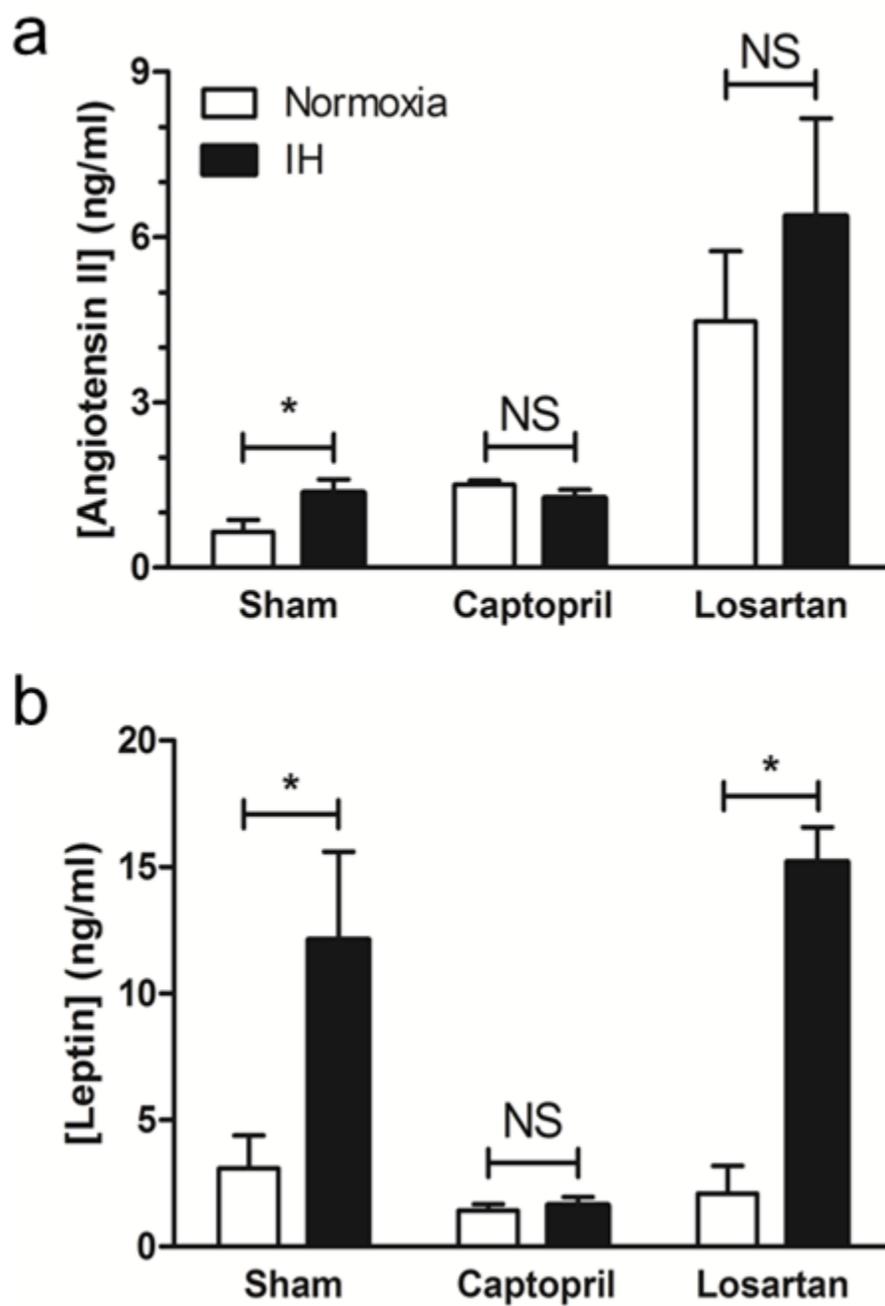
Figure 6.2 Leptin, ObRB and AT$_1$R in glomus cells in the carotid body.

Fluorescent photomicrographs of the carotid body showing glomus cells immunoreactive to leptin (a,c), Ob-Rb (e) and angiotensin type-1 receptor (AT$_1$R; b,d,f) following exposure to IH (c,d,e,f) or normoxia (a,b). Note that cells immunoreactive to leptin and Ob-Rb also contain immunoreactivity to AT$_1$R. Calibration marks indicate 25 μm.

a Leptin
Normoxia

c Leptin
IH

e Ob-Rb
IH

b AT$_1$R
Normoxia

d AT$_1$R
IH

f AT$_1$R
IH
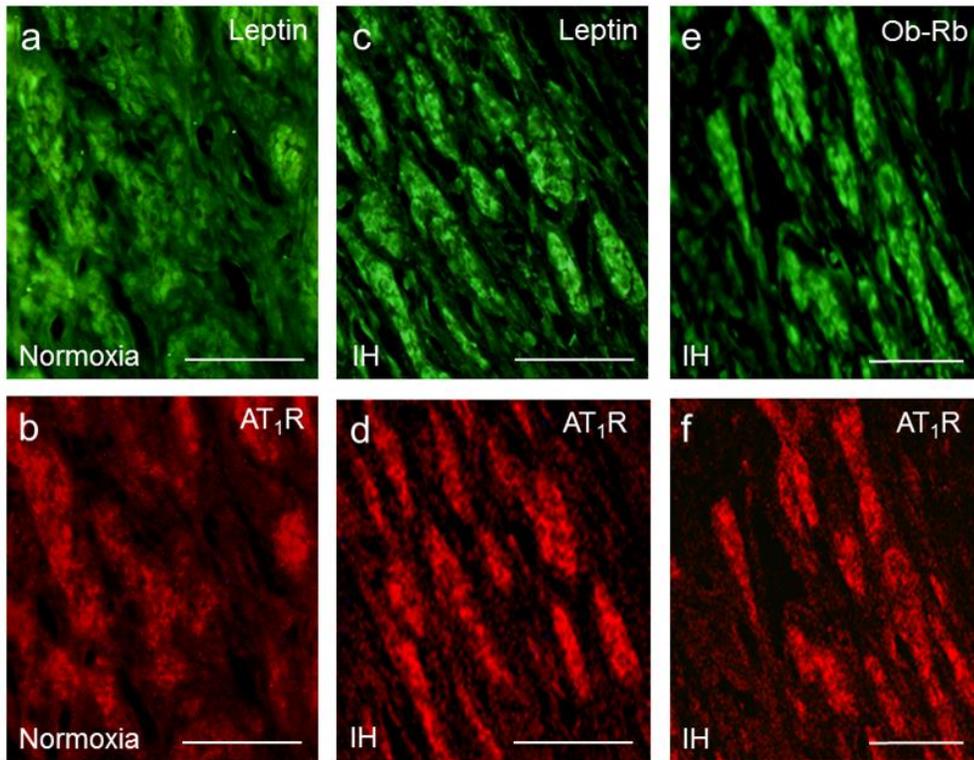
that AT_1R immunoreactivity is co-localized within the same glomus cells of the carotid body that express leptin and Ob-Rb immunoreactivity.

6.4.3 Effect of IH on AT_1R and AT_2R in carotid body

Figure 6.3 shows the effect of IH on AT_1R and AT_2R protein expression in the carotid body. When exposed to IH, the expression of AT_1R was significantly increased by about 33 % (Fig. 6.3a). However, the protein expression of AT_2R was not different following IH exposure (Fig. 6.3b). This same pattern was evident in animals that were treated with captopril (Fig. 6.3c-d). However, following losartan treatment, neither AT_1R nor AT_2R protein expression was altered in the carotid body after IH exposure (Fig. 6.3e-f).

6.4.4 Effect IH in animals treated with captopril or losartan on leptin within the carotid body

As previously reported by Messenger and Ciriello (2013), IH induced an increase in the leptin protein expression in the carotid body. Figure 6.6 shows the effect of captopril treatment on carotid body leptin protein levels following normoxia (Fig. 6.4a) or IH (Fig. 6.4b) exposure. In normoxic animals (Fig. 6.4a), captopril treatment lowered leptin levels to about 50% of non-captopril treated animals. In the IH animals, captopril (Fig. 6.4b) lowered leptin levels within the carotid body

Figure 6.3 Effect of IH on AT$_1$R and AT$_2$R within carotid body.

Western blots showing the effect of IH on AT$_1$R (a) and AT$_2$R (b) the protein expression in the carotid bodies. Note that AT$_1$R protein levels are significantly elevated following IH, whereas levels of the AT$_2$R are not altered in the carotid bodies (a-b). These effects on the AT$_1$R were maintained after captopril treatment (c-d). However, losartan treatment eliminated the effects of IH on AT$_1$R protein expression (e), and did not alter AT$_2$R (f) protein expression. *, significantly different from normoxic control animals.
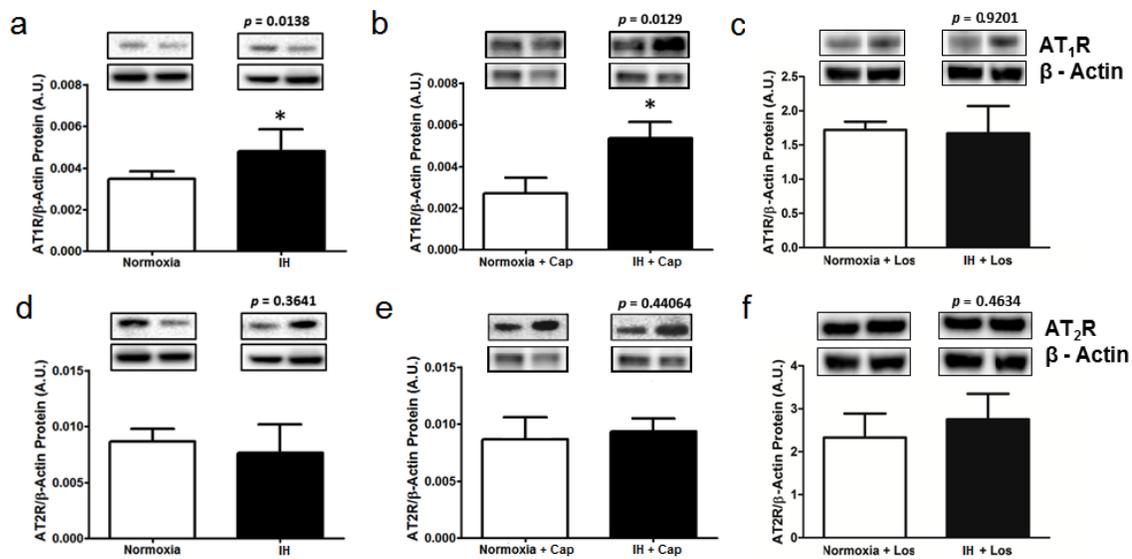
Figure 6.4 Carotid body leptin protein following IH.

Western blots showing changes in leptin protein expression within the carotid body following IH with or without captopril (Capt; a-c) or losartan (Los; d) treatment. Note that captopril treatment lowered leptin levels within the carotid body in both normoxic (Norm) (a) and the IH (b) group. In addition, the decrease in leptin following captopril treatment was greater in the IH group compared to the normoxic group (c). A similar decrease in leptin protein expression is observed following losartan treatment (d). *, significantly different from non-captopril IH treated animals.
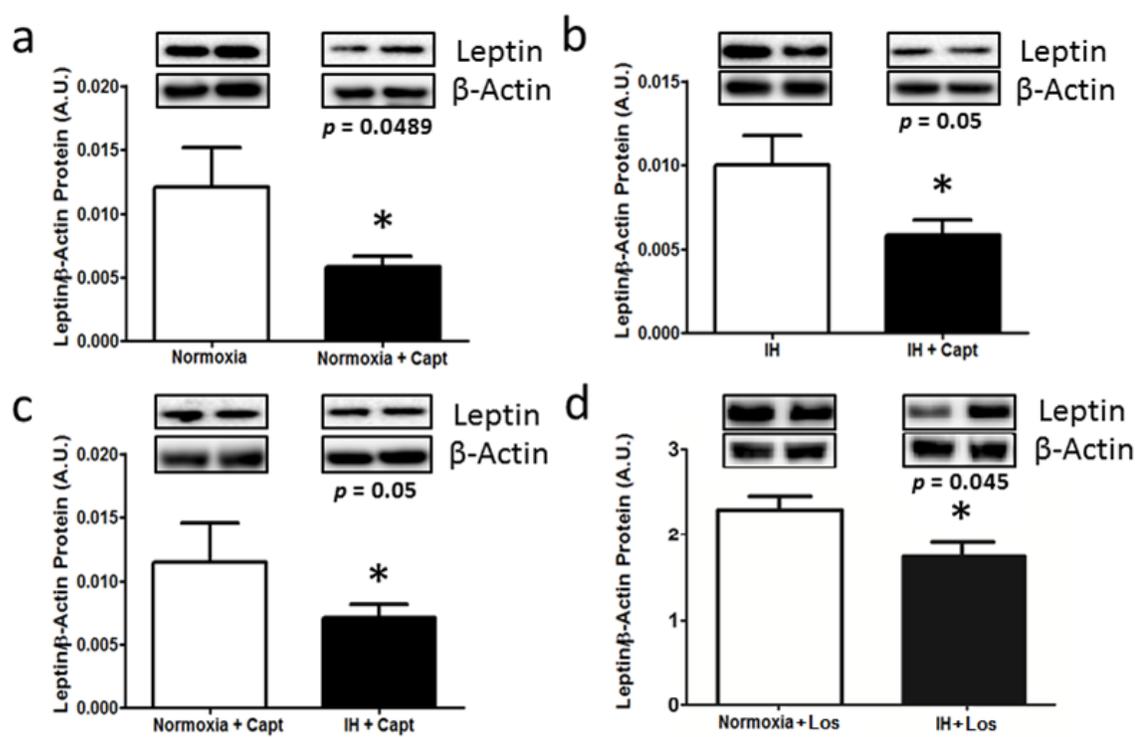
to about 58%, of those levels found within the IH non-captopril treated animals. In addition, when the effects of captopril (Fig. 6.4c) in the normoxic animals were compared to those in the IH animals, it was found that captopril lowered the leptin levels of the IH animals to about 60% of the normoxic captopril animals (Fig. 6.4c). Similarly, losartan significantly lowered carotid body leptin protein expression to IH (Fig. 6.5d; Table 6.1).

6.4.5 Effect of IH on OB-Rb and OB-R$_{100}$ protein expression within the carotid body following captopril or losartan treatment

Captopril treatment in either the normoxic controls (Fig. 6.5a) or the IH exposed (Fig. 6.5c) animals resulted in a lower carotid body OB-Rb (Fig. 6.5a and 6.5c) protein levels (31% and 44%, respectively) compared to normoxic controls, while the protein expression levels of OB-R$_{100}$ (Fig. 6.5b and 6.5d) were increased to about 100% and 140% above the levels within the non-treated normoxic (Fig. 6.5b) and IH (Fig. 6.5d) animals, respectively. Additionally, when the effects of captopril in the normoxic animals were compared to those of the IH animals (Fig. 6.6a), it was found in the IH animals that captopril raised the expression levels of the OB-Rb protein to about 140% more than the normoxic controls. On the other hand, no differences were found with the expression of the OB-R$_{100}$ protein (Fig. 6.6b) between normoxic- and IH-captopril treated animals. In contrast, both OB-Rb and OB-R$_{100}$ protein expression were not altered in the IH group compared to the normoxic controls after treatment with losartan (Table 6.1).

Table 6.1 The effect of captopril or losartan treatment on ANG II receptors and leptin signaling molecules in the carotid body following IH.

Treatment Protein	IH (*p* value)	IH + Captopril (*p* value)	IH + Losartan (*p* value)
AT_1R	↑ 0.0129	↑ 0.0138	0.9201
AT_2R	0.4406	0.3641	0.4634
Leptin	↑ *	↓ 0.05	↓ 0.0452
OB-Rb	↓*	↑ 0.0348	0.5232
OB-R100	↑ *	0.2471	0.7864
pSTAT3	No Change *	0.2219	0.8345
pERK1/2	↑ *	0.246	0.2093
SOCS3	↑ *	0.28	0.3421

All *p* values are in comparison to normoxic controls with captopril or losartan treatment. *, adapted from Messenger and Ciriello, 2013.

Figure 6.5 ObR isoforms following Capt in carotid body.

Western blots showing changes in OB-Rb (a-b) and OB-R$_{100}$ (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment lowered Ob-Rb protein levels within the carotid body in both normoxic (Norm) (a) and the IH (b) group. On the other hand, the protein levels of OB-R$_{100}$ were increased in both the normoxic (c) and the IH (d) groups. *, significantly different from non-captopril treated animals within each group.
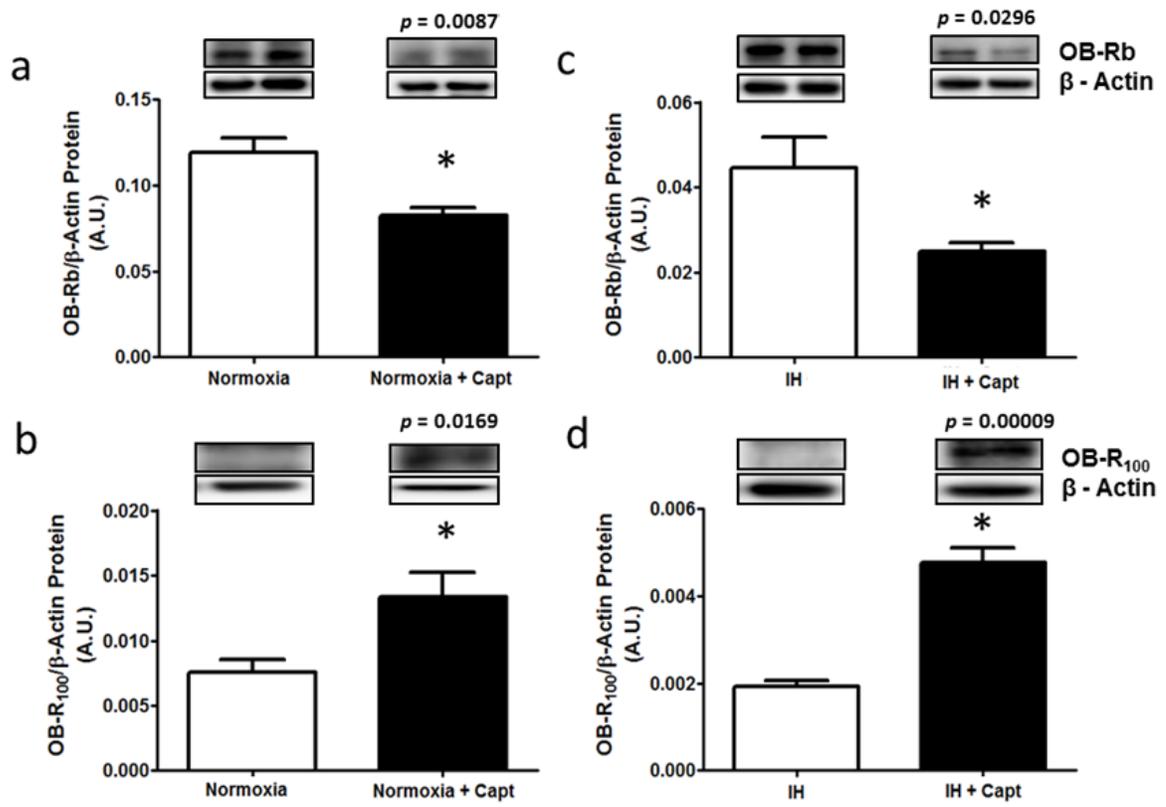
Figure 6.6 ObR isoforms following Capt: IH vs. normoxic groups.

Western blots showing changes in OB-Rb (a) and OB-R$_{100}$ (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein level of Ob-Rb was significantly (*) lower in the IH animals compared to the normoxic animals (a) treated with captopril.

a

p = 0.0348

OB-Rb

β - Actin

OB-Rb/β-Actin Protein (A.U.)

*

Normoxia + Capt

IH + Capt

b

p = 0.2471

OB-R$_{100}$

β - Actin

OB-R$_{100}$/β-Actin Protein (A.U.)

Norm + Capt

IH + Capt
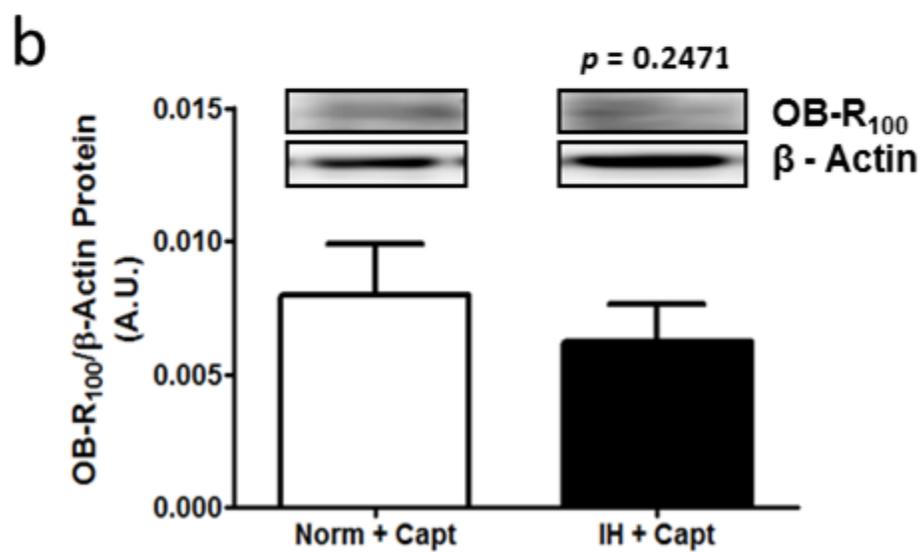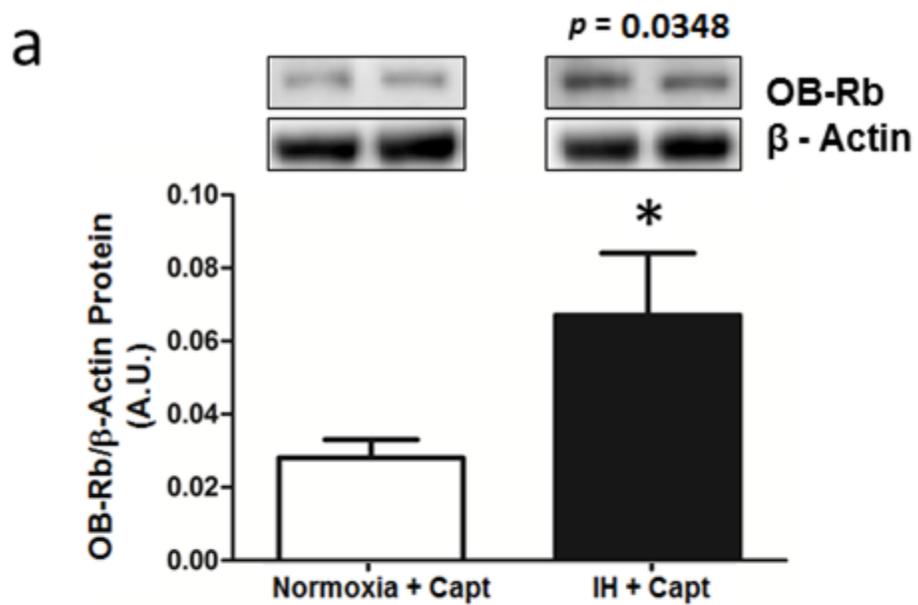
6.4.6 Effect of captopril or losartan on STAT3, pSTAT3, SOCS3 and pERK 1/2 in the carotid body after IH

Figures 6.7-6.11 summarize the effects of captopril treatment on carotid body protein expression of STAT3 (Figs. 6.7a-b and 6.8a), pSTAT3 (Figs. 6.7c-d and 6.8b), SOCS3 (Fig. 6.9a-c), and pERK 1/2 (Figs. 6.10 and 6.11a-c; Table 6.1) in normoxic and IH exposed animals. Captopril treatment in normoxic animals resulted in decreased STAT3 protein expression (Fig. 6.7a) to about 47% of non-treated group, whereas no differences were found between IH-exposed animals (Fig. 6.7b) or between normoxic and IH animals treated with captopril (Fig. 6.8a) or losartan (Table 6.1). Captopril treatment in normoxic and IH exposed animals also decreased pSTAT3 (52% and 49% of non-captopril treated animals, respectively) protein expression (Fig. 6.7c-d). However, STAT3 and pSTAT3 protein levels in the IH-captopril treated animals were not different from those in the normoxic-captopril treated animals (Fig. 6.8; Table 6.1). Similarly, pSTAT3 protein levels in the IH-losartan treated animals were not different from those in the normoxic-losartan treated animals (Table 6.1).

Captopril treatment in normoxic and IH exposed animals increased SOCS3 protein expression within the carotid body (Fig. 6.9a-b). In normoxic animals, captopril increased the expression of SOCS3 protein by almost 1036% (Fig. 6.9a), while in the IH exposed animals captopril increased the expression of SOCS3 protein by about 2490% (Fig. 6.9b). However, the protein expression level of SOCS3 in the IH captopril treated animals compared to the normoxic

captopril treated animals was not different (Fig. 6.9c; Table 6.1). Similarly, SOCS3 protein levels in the IH-losartan treated animals were not different from those in the normoxic-losartan treated animals (Table 6.1).

ERK1/2 and pERK1/2 protein levels were decreased (to about 48% and 42% of non-captopril treated animals, respectively) following captopril treatment (Fig 6.10a and 6.10c). On the other hand, neither ERK1/2 or pERK1/2 protein levels in the IH captopril treated animals were different from those in the non-captopril treated IH exposed group (Fig, 6.10b and 6.10d) or from the normoxic captopril treated groups (Fig. 6.11). Similarly, ERK1/2 and pERK1/2 protein levels in the IH-losartan treated animals were not different from those in the normoxic-losartan treated animals (Table 6.1).

Figure 6.7 Changes in STAT3 following Capt

Western blots showing changes in STAT3 (a-b) and pSTAT3 (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) lowered STAT3 protein levels within the carotid body only in normoxic (Norm) group (a). However, pSTAT3 levels were significantly (*) lower in both the normoxic (c) and the IH (d) groups after captopril treatment.
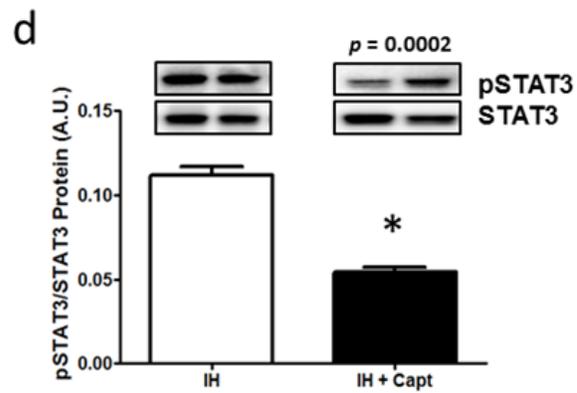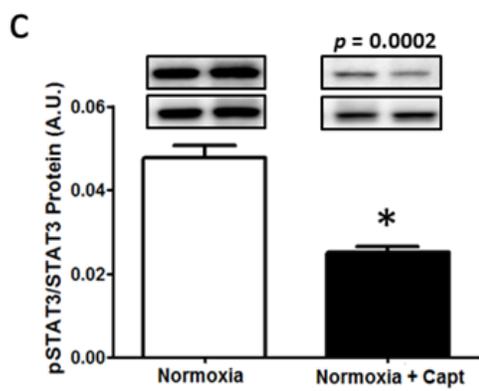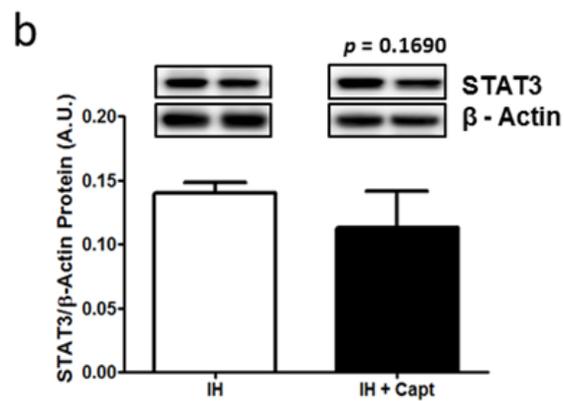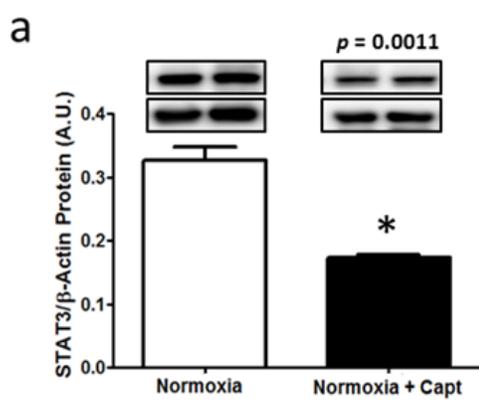
Figure 6.8 Changes in STAT3 following Capt: IH vs. normoxic groups

Western blots showing changes in STAT3 (a) and pSTAT3 (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein level of pSTAT3 was significantly (*) lower in the IH animals compared to the normoxic animals (b) treated with captopril.

a
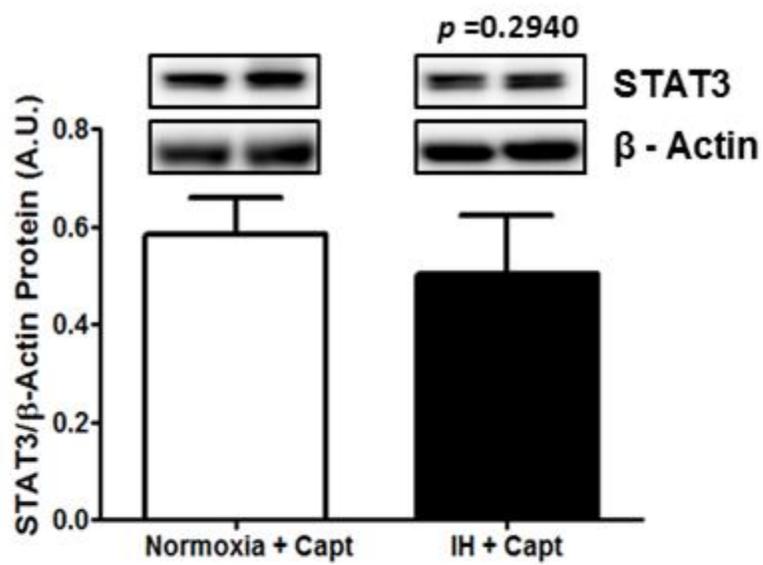
b
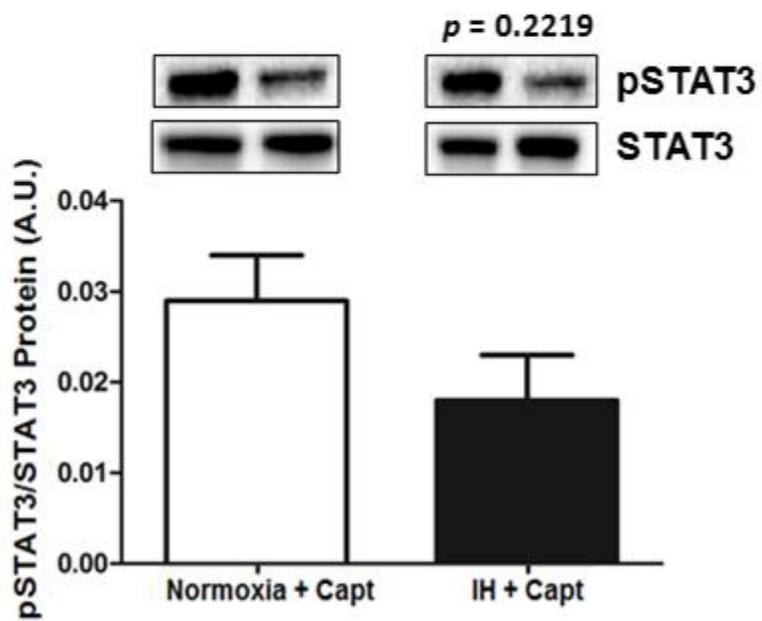
Figure 6.9 Changes in SOCS3 following IH and Capt treatment.

Western blots showing changes in SOCS3 protein expression within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) elevated SOCS3 protein levels within the carotid body in the normoxic (Norm) (a) and IH group (b). However, SOCS3 levels were significantly (*) lower in the IH captopril treated group compared to the normoxia captopril treated group (c).
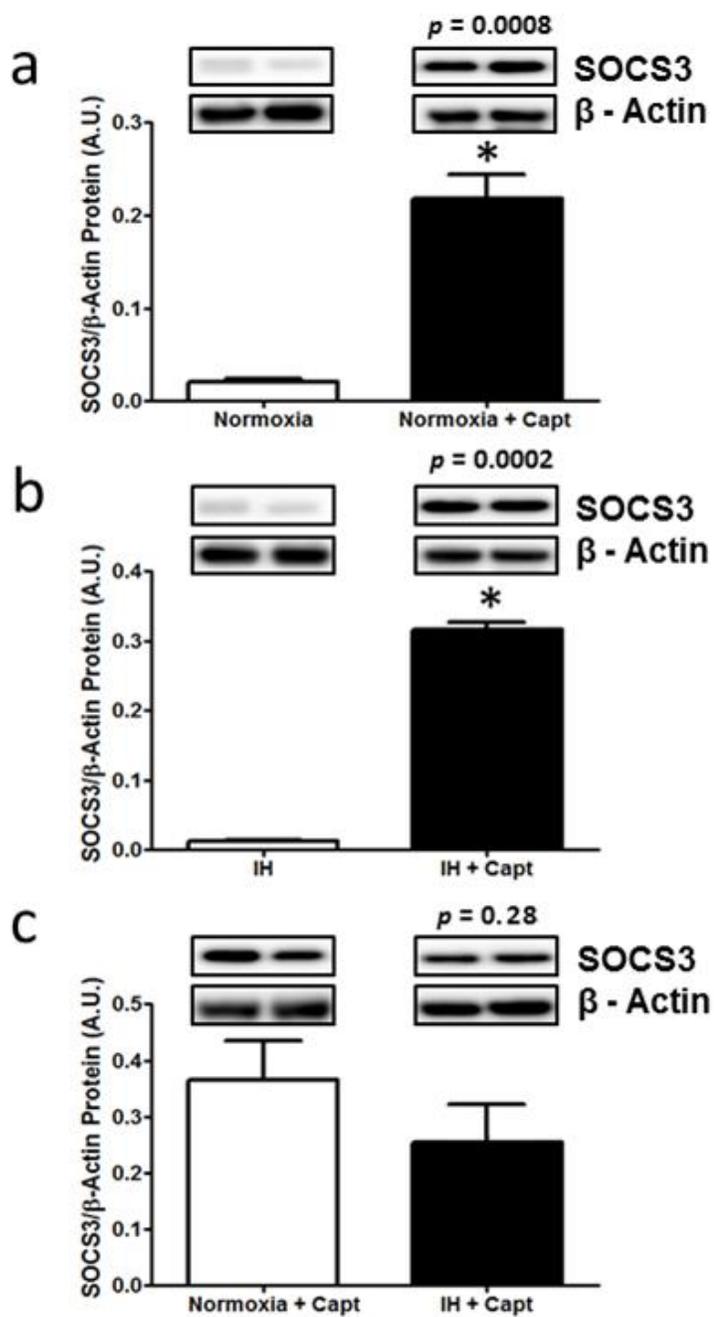
Figure 6.10 Changes in ERK1/2 following IH and Capt treatment

Western blots showing changes in ERK 1/2 (a-b) and pERK 1/2 (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) lowered ERK 1/2 and pERK 1/2 protein levels within the carotid body only in normoxic (Norm) group (a and c).
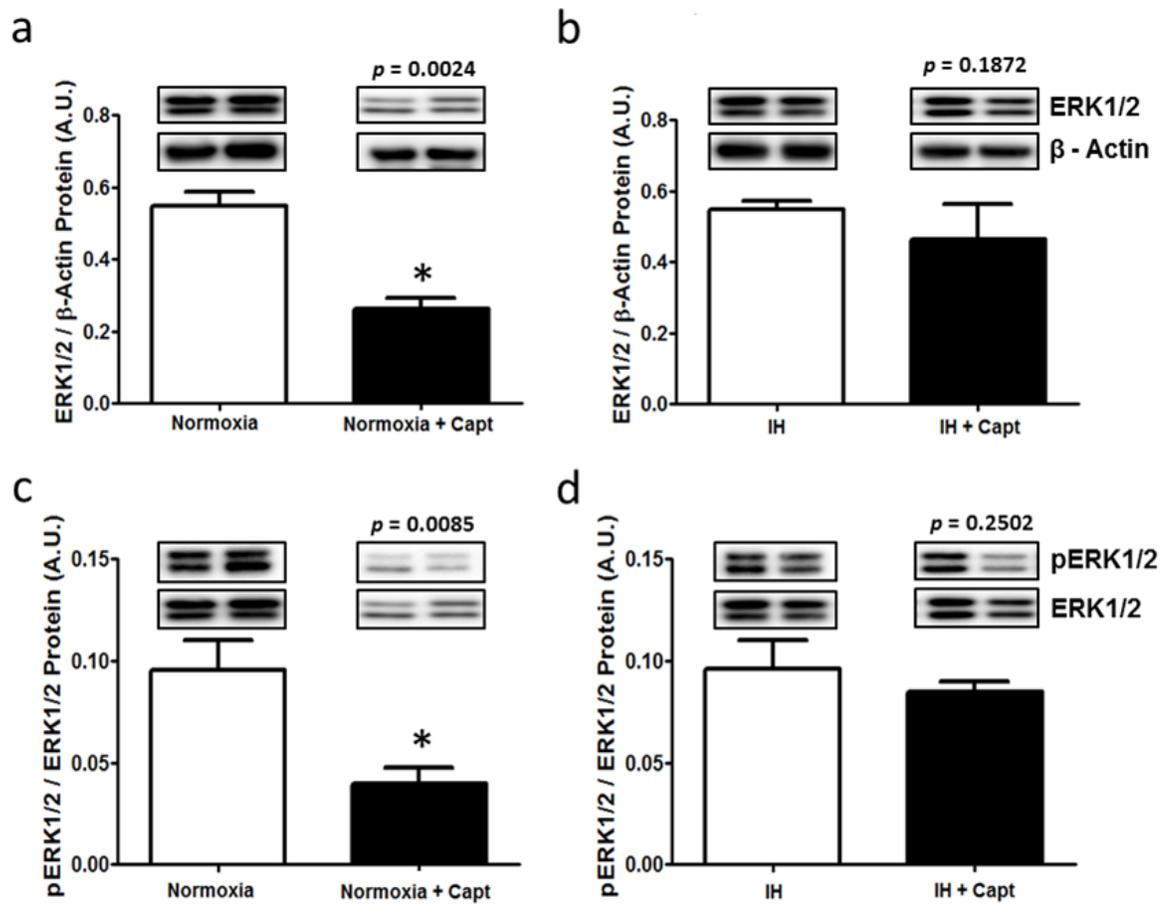
Figure 6.11 Changes in ERK1/2 following Capt: IH vs. normoxic groups

Western blots showing changes in ERK 1/2 (a) and pERK 1/2 (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein levels were not altered in either group treated with captopril.
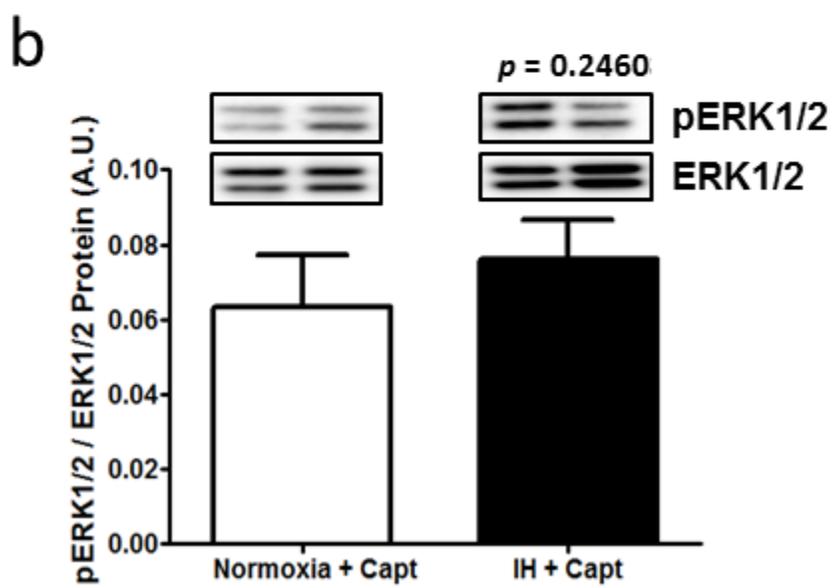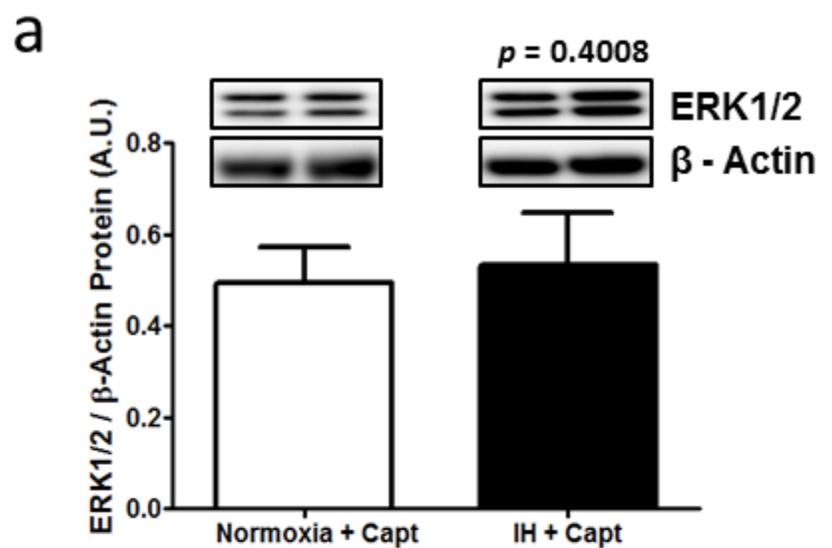
6.5 DISCUSSION

These data provide the first direct evidence that ANG II plays an important role in the regulation of leptin not only within the carotid body, but also within the circulation during IH, and that within the carotid body ANG II through activation of AT_1R contributes to changes in downstream leptin signaling during IH. These conclusions are based on the findings that within the carotid body, cells that expressed leptin and the OB-Rb also expressed the AT_1R. Additionally, inhibition of ANG II by blocking ACE following captopril treatment decreased leptin not only within the carotid body, but also the plasma levels of leptin during IH. Furthermore, the decrease in available leptin to the carotid body was accompanied by an increase in the OB-Rb protein expression in captopril treated animals, but no change in the losartan treated animals. This coupled with the lower carotid body levels of leptin during IH in the captopril and losartan treated animals suggests a dampening of the entire leptin signaling system within the carotid body by the lack of ANG II and activation of AT_1R. This suggestion is consistent with the observation that both captopril and losartan treatment resulted in an inhibition of the downstream mediators of leptin signaling within the carotid body following IH. Finally, contrary to the increase in OB-Rb protein expression levels following captopril treatment, $OB-R_{100}$ protein levels in the carotid body were increased suggesting either an increase in transport of leptin out of cells or an increase in leptin degradation within the cells. Taken together, these data can be interpreted to suggest that ANG II effects on carotid body chemosensitivity may be mediated in part through the activation of the carotid

body leptin system, and that ANG II signaling is also critical for the maintenance of circulating levels of leptin in normoxia and during IH.

The finding that ANG II influences leptin activity and requires functional leptin receptors is supported by the observation that AT_1R blockade results in a decreased body mass index and food intake, but has no effect in obese Zucker rats with a genetic mutation in the leptin receptor (Muller-Fielitz et al., 2011). In addition, it has been shown that the brain RAS facilitates renal and brown adipose tissue sympathetic nerve responses to leptin (Hilzendeger et al., 2012). However, the finding that plasma leptin levels induced by IH are not altered after losartan treatment, whereas carotid body leptin is inhibited suggests that not all leptin effects are mediated by the selective activation of the ANG II AT_1R.

The finding of a decrease in circulating leptin following ANG II inhibition is consistent with earlier data suggesting that ANG II signaling stimulates not only leptin production, but also leptin secretion (Cassis et al., 2004; Premaratna et al., 2012). Considering that the main production/secretion site of leptin into the plasma is adipose tissue (Lonnquist et al., 1996; Caro et al., 1996), this study provides further supports the suggestion that the leptin system may be in part under the control of ANG II. This latter suggestion is also consistent with the observation in a mouse model deficient in the AT_1Ra isoform, in which plasma leptin concentrations were found to be reduced when compared to wild-type (Kaneko et al., 2011).

Carotid body leptin levels were also found to be decreased in animals treated with captopril during both normoxia and IH conditions. This finding is similar to that observed within adipocytes taken from rats treated with captopril, in which both circulating leptin and leptin release was decreased (Cassis et al., 2004). Additionally, incubation of adipocytes with ANG II resulted in an up-regulation of leptin mRNA, as well as leptin secretion (Cassis et al., 2004) while in ventricular myocytes, ANG II promotes *de novo* synthesis and secretion of leptin (Rajapurohitam et al., 2006). Furthermore, blocking OB-Rs in these cardiomyocytes prevented the hypertrophic effects ANG II normally has on these cells suggesting that leptin may mediate effects normally attributed to direct ANG II action (Rajapurohitam et al., 2012). Finally, in human vascular smooth muscle cells, it has been previously found that increased ANG II results in increased leptin protein and mRNA expression (Shyu et al., 2012). Thus, it is not unreasonable to suggest that leptin may be involved in mediating some of the changes in carotid body chemosensitivity normally attributed to ANG II (Gomez-nino et al., 1990; Eyzaguirre and Zapata, 1968; Bock, 1980).

Interestingly, the increased leptin protein and mRNA expression observed in human vascular smooth muscle cells as a result of the elevated ANG II has been attributed to increases in reactive oxygen species (Shyu et al., 2012). The IH induced changes in the carotid body chemosensitivity have been suggested to be related to an enhanced reactive oxygen species production (Pawar et al. 2009; Iturriaga et al., 2009). In addition, the IH effects associated with increases in superoxide have been shown to be blocked by losartan, a specific AT_1R

blocker (Marcus et al., 2010). Therefore, taken together, these data suggest a possible mechanism by which ANG II signaling may modulate ROS activity and subsequently leptin activity within the carotid body.

Captopril treatment in both normoxia and after IH reduced protein levels of OB-Rb compared to their respective controls. This finding suggests that ANG II plays an important role in the regulation of Ob-Rb, although due to the low levels of available leptin both locally and from the circulation following captopril treatment, ligand-induced endocytosis seems unlikely (Bennett et al., 1998; Uotani et al., 1999). The short-form leptin receptor displayed increased levels of protein expression in the carotid bodies following captopril-treated animals in both the normoxic and IH conditions. Generally, OB-Ra is believed to be involved in leptin transport, specifically across the blood-brain barrier (Banks et al., 1996; Golden et al., 1997; Bjorbaek et al., 1998). One potential explanation may be that the reduced leptin availability stimulates OB-Ra protein expression to aid in leptin secretion from glomus cells. Additionally, the OB-Ra has been suggested to function in the degradation process of leptin (Iida et al., 1996; Merabet et al., 1997; Sharma et al., 1997) and may provide one mechanism through which the decreases seen in carotid bodies occurs. However, the functional significance of ANG II effects on Ob-Ra is unknown.

The lower protein levels of leptin and OB-Rb in the carotid bodies after captopril treatment is consistent with the decreases in activation of STAT3. In normoxic and IH conditions, captopril treatment reduced pSTAT3 levels, a downstream signaler involved in OB-Rb activation (Elmquist et al., 1997;

Fruhbeck, 2006; Wang et al., 1998; Huo et al., 2006, 2007). The down-regulation of both the ligand and the receptor would be expected to result in a decrease in activation of the signaling pathway (Elmquist et al., 1997; Fruhbeck, 2006; Wang et al., 1998; Huo et al., 2006, 2007). One other potential explanation for the decrease in pSTAT3 levels found in captopril treated animals is the decrease in ANG II levels as ANG II signaling, through AT_1R, can activate the JAK/STAT pathway resulting in phosphorylation of STAT3 (Ji et al., 2012; Omura et al., 2001). However, it has also been shown that leptin injections resulted in up-regulation of pSTAT3 protein expression (Messenger et al., 2012), and thus this decrease after ANG II inhibition most likely occurs at least in part through decreased leptin signaling.

Consistent with the decrease in pSTAT3 is the concomitant rise in SOCS3 levels. SOCS3 acts as a negative feedback loop on JAK/STAT signaling (Bjorbaek et al., 2000; Fruhbeck et al., 2006). The decrease in pSTAT3 accompanied by the SOCS3 rise would be expected in the IH condition, which has previously been shown to stimulate the JAK/STAT pathway (Messenger et al., 2012; Messenger & Ciriello, 2012). In some cell types, ANG II has been known to stimulate the JAK/STAT pathway which results in increased SOCS3 expression (Calegari et al., 2003; Ji et al., 2012; Omura et al., 2001). However, as to the reason why such a profound increase was found in both the normoxic and IH condition is unknown.

In addition to the JAK/STAT pathway, OB-Rb signaling can also activate the MAPK cascade (Bjorbaek et al., 1997; Banks et al., 2000) and thus activation

of ERK1/2 is an indicator of pathway activation. In normoxic animals, captopril treatment resulted in a decrease in pERK1/2 protein levels, however no changes were observed in the IH animals. This finding suggests during IH, other mechanisms in addition to leptin (Bjorbaek et al., 1997; Banks et al., 2000) or ANG II (Li et al., 1998; Nakai et al., 2012) may activate ERK1/2.

In summary, this study has demonstrated that the RAS modulates the leptin system not only in the circulation, but also within the chemosensitive carotid body. Inhibition of ANG II by ACE results in a dampening effect on circulating leptin levels as well as local carotid body leptin protein levels. There is also a decrease in the OB-Rb and downstream indicators of OB-Rb activation, and this appears to be mediated through an interaction with the activation of the AT_1R. Angiotensin signaling through the AT_1R is critical for this chemosensitivity by the carotid bodies (Marcus et al., 2010), and given its co-localization with OB-Rb and leptin within the glomus cells suggests that AT_1R has a stimulatory effect on the carotid body leptin system and ANG II chemosensitive role may be mediated in part through leptin signaling.

6.6 FOOTNOTES

6.7 REFERENCES

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CHAPTER 7

SUMMARY AND CONCLUSIONS

7.   CHAPTER 7

7.1 SUMMARY OF MAJOR FINDINGS

This thesis examined the effects of intermittent hypoxia (IH) exposure on physiological responses related to energy balance and cardiovascular regulation in rats. Physiological alterations were observed following both acute and chronic exposure to IH, and included changes in neural pathways associated with these physiological systems. The role of leptin signaling in these pathways was an underlying theme, and was altered in both acute and chronic models, an important factor in mediating many of the observed effects.

In Chapter 2 of this thesis, I wanted to evaluate alterations in body energy balance associated with exposure to acute IH. This study was performed given that chronic models of IH have significant, detrimental metabolic alterations and that OSA patients also present with altered metabolism and body energy balance (Martinez et al., 2008; Dempsey et al., 2010). The role of leptin in these major changes was also determined using a leptin-deficient transgenic rat in Chapter 3. These KILO animals have the leptin gene removed through zinc-finger nuclease technology (Vaira et al., 2012). Body energy balance is significantly altered in wild-type rats exposed to IH, displaying a negative body energy balance, including body weight loss likely due to decreased food intake. Interestingly, the body energy expenditure appeared to be reduced in these animals, given that food conversion efficiency and locomotion were both decreased compared to normoxic controls. These alterations were associated with a significant elevation in circulating leptin, concomitant with reduced adipose leptin content. Circulating

adiponectin was reduced, whereas adipose adiponectin content was elevated. Leptin:adiponectin ratio was elevated in animals exposed to IH, as a result. The ARC of animals was isolated and protein expression of pSTAT3 was shown to be higher in animals exposed to IH, suggestive of activation of a cytokine receptor dependent on the JAK2/STAT3 signaling pathway. POMC was also higher in the ARC of IH animals, and this was mirrored by an increased number of POMC cells expressing immediate early gene product immunoreactivity. When leptin-deficient KILO rats were used, none of these differences were observed between IH and normoxic exposed animals, suggesting that leptin is a necessary component of mediating the body energy balance phenotype in animals exposed to IH.

Since the role of leptin in mediating the effects of IH on body energy balance appeared to be critical, we looked at the role of chronic IH in body energy balance and whether a state of leptin resistance could potentially be induced in Chapter 4. Certainly animals exposed to 95 days of IH had reduced body weight compared to controls, but by the end of the study period, animals exposed to IH were gaining no more body weight than at the start of the period, compared to a reduction in body weight gain in controls, an effect mirrored by energy utilization. These changes were concomitant to an elevation in food intake in CIH animals over the study period, whereas normoxic animals had a significant reduction in food intake over the same time. This may be suggestive of a physiological manifestation of leptin resistance. Leptin sensitivity was measured directly, and it was shown that CIH animals were resistant to leptin

compared to normoxic controls. Animals had elevated leptin/epididymal fat pad mass, an important factor given the alterations observed in body energy balance. No changes in locomotion were observed. CIH animals exhibited no differences in the protein expression level of neither ObRB, nor ObR100, nor pSTAT3, though pERK1/2 was curiously elevated. POMC protein was decreased and SOCS3 increased in the ARC, suggesting that leptin resistance may occur following CIH through SOCS3 induction, and POMC is reduced as a result of the decreased leptin signaling. This study suggests that IH may be able to induce a state of positive body energy balance over long-term exposures.

In Chapter 5, the role of acute, short-term and chronic IH on hemodynamic measures were assessed and associated with neuroplastic alterations within the NTS region of the brainstem. Hypertension without concomitant alterations in heart rate was observed in animals following 95 days of IH, but not at any other time point. The basal gain of the baroreflex was also reduced following this exposure. It was observed that specific alterations in the TrkB-BDNF signaling system change over time, which may be the result of differential leptin signaling. Interestingly, the amount of synaptophysin within the NTS region was reduced following IH, indicative of a loss of synapses within the region. However, in acute IH this effect was still observed in KILO leptin-deficient animals, suggesting leptin does not play a role in this effect. The neurite growth factor GAP-43 was reduced following CIH, suggesting a potential reduction in cardiorespiratory afferent neurites or potential for neurite sprouting within the NTS region. We believe this may be a novel mechanism mediating hypertension following IH, an effect

supported by other groups observing a reduction in glutamatergic synapses following IH (Almado et al., 2012).

Finally in Chapter 7, the role of angiotensin II in mediating leptin signaling within the carotid body following IH was assessed. This was done by exposing animals to acute IH following angiotensin converting enzyme (ACE) inhibition or blockade of the type 1 angiotensin II receptor (AT_1R), and served as a follow-up to initial findings describing the effects of acute IH on leptin signaling in the carotid body (Messenger and Ciriello, 2013). Here, we show that leptin protein within the carotid body is reduced in animals exposed to IH compared with controls in both conditions of reduced angiotensin II signaling. The treatments also prevented all the alterations observed in leptin signaling within the carotid body following IH under sham conditions, suggesting that angiotensin II plays a critical role in mediating the effect of leptin at the carotid body during IH. Likewise, it was observed that circulating leptin concentrations were not different in IH animals following ACE inhibition, but were elevated during AT_1R blockade following IH, similar to sham animals. This suggests that leptin secretion following IH is dependent on angiotensin II acting through non-AT_1Rs.

7.2 LIMITATIONS AND FUTURE STUDIES

The animal model of IH used in these studies under both acute and chronic exposures is a sleep-independent model, which is applied during the rest phase of the sleep/wake cycle of the rats used. Due to the sleep-independent nature of

this exposure, it could be that for some short periods of time animals were being exposed to IH while actually awake. The effect this may have on the outcomes of these studies is not known, but may be negligible since studies using IH exposure during an aroused state in humans showed effects consistent with that observed in patients with obstructive sleep apnea (OSA; Louis and Punjabi, 2009). Likewise, alterations in oxygen availability in patients with OSA may persist throughout the waking hours (Shiota et al., 2013).

In addition to potential inconsistencies of hypoxia between the model of IH utilized and OSA, it has previously been shown that rodents exposed to IH experience an elevated ventilator response, resulting in eucapnea and no alterations in intrathoracic pressure (Fletcher et al., 1992). In patients with OSA, both hypercapnemia and reductions in intrathoracic pressure similar to that seen in the Muller manoeuvre have been noted during apnea (Dempsey et al., 2010). This is the result of difference between the inductions of IH experimentally and clinically. In the model of IH, environmental oxygen levels are altered, but the airway of the animal or subject remain intact. This occurs without a change in environmental pressure or carbon dioxide, and thus breathing is free to go unimpeded and without any change in carbon dioxide within the lungs. In contrast, a reduction in airway flow during OSA results in reduced gas exchange and unsuccessful attempts to breathe during apnea (Dempsey et al., 2010). Since these models do not mimic OSA, but rather the specific IH component of OSA, is precisely the reason that models of IH are not described as models of OSA, and so differences between the model and the disease are important to

take into consideration. Despite this, it is believed that the cardiovascular and metabolic responses in OSA are largely due to the observed IH. The neurocognitive components appear to be largely due to disruption in sleep architecture and IH. Interestingly, it has been determined in rodent models that hypercapnea does not alter responses to IH (Dempsey et al., 2010).

Despite the overwhelming contributions of IH in exploring mechanistic and causal roles of OSA-induced IH to other disease states, there are other unresolved issues in modelling OSA with current IH models. The most important of which, is the sudden nature of IH exposure. OSA is a chronic, progressive disease that can persist for a significant portion of an individual's life. Most current models of CIH involve exposing rodents to IH with an apnea-hypopnea index (AHI) within the moderate-severe range immediately. This massive change in oxygen availability is likely a massive insult to the homeostasis of an animal, and does not mimic what is observed in OSA. In an attempt to resolve this issue, I have proposed a model of CIH that includes a progressive component, increasing hypoxic exposure to a plateau level that coincided with that normally exposed in our CIH model. This so-called ramped IH model consists of starting animals with an AHI of 1 event/h, with progressive steps of AHI by 1 event/h every four days. Animals are then exposed to CIH in the plateau phase for 28 days, for a total hypoxic exposure of 96 days (on par with our 95 day CIH model). I believe this model will allow for appropriate adaptation in neural signaling networks, such as the carotid body afferent system and sympathetic and phrenic efferent systems. This model must be further studied to validate these claims,

and determine the appropriateness for use in better modelling the chronic, yet dynamic exposure to IH experienced in OSA. On this topic, it may also be advisable that models of CIH be utilized for longer time periods. Given that rodents exposed to 95 days IH developed leptin resistance and had small initial alterations in food intake and body weight changes, it would be better suited to observe these models over long periods of time to determine the pathophysiological manifestation of obesity and/or metabolic syndrome. As well other, currently unstudied pathologies and their association to CIH such as heart failure and sudden cardiac death could also be studied over longer exposure times.

7.3 SIGNIFICANCE OF RESEARCH AND CONCLUSIONS

OSA represents a significant disease burden to both patients and healthcare systems globally. Given the associations of OSA with other diseases such as obesity and cardiovascular disease, the time has come to determine the true role OSA has in mediating mechanisms that lead to, and are part of, these deadly disease states. Understanding the factors that link these diseases is important for 1) an understanding of the importance of diagnosing and treating OSA, 2) mechanisms which may be targeted by OSA treatment, 3) the development of novel treatment approaches to OSA, and 4) the development of novel treatment approaches for OSA-related pathologies.

Clinical studies have shown that obesity is an important risk factor for the development of OSA, and patients with OSA tend to gain weight compared to normal individuals (Phillips et al., 2000). Likewise, OSA patients have more trouble losing weight than normal individuals. These findings form the basis for a bidirectional relationship between OSA and body weight gain. For the first time, body energy balance within animals has been assessed in a model of CIH. My study has shown that a primary and potentially causative hallmark of obesity is induced by CIH. In this study, leptin resistance occurs despite a reduction in body weight gain, though is accompanied by an increase in food intake over the exposure time, compared to controls. This may provide a mechanism by which body weight gain and obesity could be induced by the CIH observed in patients with OSA. Likewise, most of the short-term effects of IH on body energy balance are due to leptin signaling. This is concomitant to the finding that hypoxia and OSA in humans increases circulating leptin. Acute IH causes changes such as an alteration in leptin:adiponectin ratio, which is believed to be an important variable in cardiovascular disease development (Kappelle et al., 2012), though this measure has not been assessed in OSA patients.

Another common comorbidity with OSA is hypertension. My studies suggest a potential novel mechanism by which neuroplastic alterations in the nucleus of the solitary tract is associated with hypertension in animals exposed to IH. The development of hypertension in patients with OSA is often described as resistant in nature. This suggests that classical mechanisms of blood pressure elevation, which are largely targeted by drugs, are not likely the mechanisms

mediating the hypertension-inducing effects of OSA. Instead, new approaches to hypertension must be addressed in complicated forms of secondary resistant hypertension, such as the one induced by the CIH model. It cannot be ruled out that selective leptin resistance, which permits the activity of the hypertensive effects but inhibits the satiety effects of leptin, played a role in mediating this hypertension. Likewise, the alterations observed in neuroplastic markers, especially considering that leptin deficient animals do not develop the same alterations in some of the markers of neuroplasticity, may also be the result of leptin acting selectively in this area to drive blood pressure. Nonetheless, these potential pathways warrant a closer look in additional studies to determine the role of these changes following IH in hypertension.

Finally, in Chapter 4 of this thesis I have shown that leptin signaling pathways within the carotid body are regulated by the AT_1R, while the IH-dependent release of leptin is regulated by angiotensin II through non-AT_1R. The clinical relevance of this data is not obvious, but these effects may be important in suggesting a role for a complex interaction between leptin and angiotensin II both peripherally and specifically within the carotid body. The functional effects of this interaction are unknown, but it is known that angiotensin II is an important modulator of the carotid chemoreceptor reflex (Marcus et al., 2010), and so leptin may interact with this to potentiate hypoxic responses. This is important, as data suggest a potentiation of carotid body signaling and sympathetic nervous drive following exposure to IH (Dick et al., 2007).

Collectively, the studies in this thesis provide many new avenues for research into cardiometabolic diseases associated with IH exposure, presumably to determine mechanism of diseases associated with OSA. Addressing these mechanisms may provide novel therapeutic targets in addressing the disease burden associated with OSA. This disease burden will become increasingly important as OSA increases in prevalence within our population.

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APPENDIX

APPENDIX 1 ANIMAL USE PROTOCOL APPROVAL

Western

AUP Number: 2008-030-04
PI Name: Ciriello, John
AUP Title: Central Mechanisms In The Pathogenesis Of Hypertension; Leptin Links Obesity, Hypertension And Sleep Apnea; Role Of Sex Steroids In Central Regulation Of Arterial Pressure

Approval Date: 03/11/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Central Mechanisms In The Pathogenesis Of Hypertension; Leptin Links Obesity, Hypertension And Sleep Apnea; Role Of Sex Steroids In Central Regulation Of Arterial Pressure

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2008-030-04::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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Experience

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Positions Held

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Awards and Recognition

Awards

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Ontario Hypertension Society's Trainee Education Grant, 2009, 2010.
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Schulich Graduate Research Scholarship, awarded by the Schulich
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Ontario Graduate Scholarship, awarded by the Ontario Ministry of
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Canadian Obesity Network Summer Bootcamp Travel Award,
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CIHR – Institute of Circulatory and Respiratory Health Travel Award,
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**George W. Stavraky Teaching Scholarship in Physiology and
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Gordon J. Mogenson Research Scholarship, awarded by Department
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$1 000.

Publications

Papers Accepted for Publication (8)

Moreau JM and Ciriello J. 2013. Effects of acute intermittent hypoxia on energy balance and hypothalamic feeding pathways. Neuroscience 253:350-360.

Moreau JM and Ciriello J. 2013. Nesfatin-1 induces Fos expression and elicits dipsogenic responses in subfornical organ. Behav Brain Res 250:343-50.

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Papers Submitted for Publication (4)

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Moreau JM, Messenger SA, and Ciriello J. 2013. Effects of angiotensin II on leptin and downstream leptin signaling in the carotid body following acute intermittent hypoxia. Neuroscience. **NSC-13-1392, October 8, 2013.**

Moreau JM and Ciriello J. 2013. Chronic intermittent hypoxia induces leptin resistance in the rat. J Neuroendocrinology. **JNE-13-0165-OA, October 28, 2013.**

Moreau JM, McCoy A, Jones DL, Ciriello J**.** Effects of intermittent hypoxia on body energy balance and hypothalamic feeding pathways are abolished in the leptin-deficient *KILO* rat. Brain Res. **BRES-D-13-01522, November 16, 2013.**

Papers in Process

Moreau JM, Messenger SA, Miles A and Ciriello J. Intermittent hypoxia induces alterations in stanniocalcin-1 protein expression throughout the carotid chemoreflex neural circuit.

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Accepted Abstracts (selected)

Total Primary Author: 15

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Professional Activities

Professional Affiliations

Hypertension Canada, Student Member, 2011

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Conferences Attended

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Neuroscience 2009, Society for Neuroscience, Chicago, IL; October 17-21, 2010.

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SONA 2010, Southern Ontario Neuroscience Association, St. Catherines, ON; May 7, 2010.

Ontario Hypertension Society Annual Spring Conference 2010, Ontario Hypertension Society, Alliston, ON; May 28-30, 2010.

Neuroscience 2010, Society for Neuroscience, San Diego, CA; November 13-17, 2010.

Obesity Bootcamp, Canadian Obesity Network with Canadian Institutes of Health Research and Universite Laval, Duchesnay, QC; July 17-24, 2011.

Neuroscience 2011, Society for Neuroscience, Washington D.C.; November 12-16, 2011.

Experimental Biology 2012, Federation of American Societies for Experimental Biology, San Diego, CA; April 21-25, 2012.

Canadian Institutes of Health Research – Institute of Circulatory and Respiratory Health's Young Investigator's Forum, Montreal, QC; June 4-6, 2012.

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