EFFECT OF ACUTE SLEEP RESTRICTION ON THE CONCENTRATION OF SERUM SOLUBLE LEPTIN RECEPTOR IN HEALTHY ADULT FEMALES

A Thesis

presented to

the Faculty of Natural and Applied Sciences

at Notre Dame University-Louaize

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

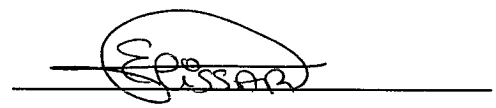
ELISSAR ELIAS AZZI

JUNE 2018

Thesis Release Form

☑ I Eissar Elias Azar , authorize Notre Dame University-Louaize to supply copies of my thesis to libraries or individuals on request.

☐ I _____ , do not authorize Notre Dame University-Louaize to supply copies of my thesis to libraries or individuals on request.



Signature

23/7/18

Date

Notre Dame University - Louaize

Faculty of Faculty of Natural and Applied Sciences

Department of Biology

We hereby approve the thesis of

Elissar Elias Azzi

Candidate for the degree of Master of Science in Biology

Dr. Colette Kabrita-Bou Serhal	June 26th, 2018 Supervisor, Chair
Dr. Robert Dib	June 26, 2018 Committee Member
Dr. Diala El Khoury	June 26th, 2018 Committee Member

Dedications

To Dr. Colette Kabrita-Bou Serhal. You are and always will be more than just an instructor to me. The amount of knowledge present in you and your wit is the reason why I am pursuing higher education and pushing forward in this field, with the hope that someday I can become the person you are today.

To Dr. Tanos Hage .Because of you, I now know what the word passion means. I hope one day I can inspire people around me the way your passion, teaching and support inspired me.

To my family. You are a blessing in disguise. It is because of your annoyance that I am the person I am today.

To Cookie. You keep me sane.

Acknowledgements

I thank **Dr. Colette Kabrita-Bou Serhal** for her never-ending support and knowledge. None of the work done would have been possible without her guidance and supervision.

I also thank **Dr. Robert Dib** for his valuable insight and suggestions that gave additional depth to this study.

Thanks to **Dr. Tanos Hage** for his excellent teaching that helped come up with the proposal.

Thanks to **Mrs. Nada Maalouf** for her infinite patience and help with every protocol and technique.

Thanks to **Laboratoires Saint Georges-Zalka** for their help with electrochemiluminescence immunoassay for the detection of cortisol levels.

Thanks to **Dr. Re-mi Hage** for her help with statistical analyses.

And a special thanks to my friends and family, who doubled as lab assistants and tolerated the endless piles of articles around me.

Abstract

Sleep restriction is now considered to be a worldwide epidemic, as over 20% of people around the world are sleep deprived. Although sleep deprivation is now an overlooked feature of modern societies, it entails considerable adverse effects on physiological systems, one being energy homeostasis. A corpus of research has demonstrated that leptin, a key hormone that conveys the body's energy status to the brain, decreases after sleep restriction, which may partly account for the associated alteration in body metabolism; however little information is available regarding the underlying physiological mechanisms. The aim of the present study was to investigate the diurnal serum profile of both leptin and its soluble receptor - sOB-R - before and after acute sleep restriction. Five healthy adult females (20–30 years old; BMI 22-26) were maintained on an 8-hour sleep/wake schedule (bedtime at 12:00 AM) for one week [baseline or control (C) period]. This was followed by 2 consecutive nights of a 4.5-hour nocturnal sleep opportunity [sleep restriction (SR), bedtime at 3:30 AM]. Throughout the duration of the study, sleep parameters were objectively measured using wrist actigraphy and participants were asked to keep a food and sleep diary. For the determination of circulating free hormone and soluble receptor levels, blood and saliva samples were collected from each subject at 4-hour intervals (starting 8:00 AM) on the days before and following SR. Results showed that the diurnal serum levels of free leptin and sOB-R were out-of-phase (discordant) with respect to each other before and following SR. Leptin levels were significantly elevated in the early morning, mid-afternoon, and midnight; while sOB-R levels were high around noontime and late evening. SR did not affect the diurnal serum pattern of both free hormone and its receptor, but rather altered their average daily concentrations. That is, SR caused a significant decrease in circulating free leptin levels (SR, 20.94 $\pm$ 1.71 ng/ml vs. C, 25.71 $\pm$ 1.71 ng/ml; $P < 0.01$) and a concomitant increase in

sOB-R levels relative to baseline (SR, 24.39 $\pm$ 1.21 ng/ml vs. C, 19.79 $\pm$ 1.643 ng/ml; $P < 0.01$). These findings may suggest that SR works on modulating circulating free leptin levels, and thus its regulatory role on body metabolism (or energy homeostasis), via regulating sOB-R levels. The putative regulatory role of leptin on its soluble receptor may have valuable implications on therapeutic approaches that may consider sOB-R as a potential target for countering body weight disorders and metabolic disturbances associated with disturbed sleep.

Key words: adult females, circadian rhythm, energy homeostasis, leptin, metabolism, sleep deprivation, sOB-R.

Table of content

List of Tables and Figures

List of Abbreviations

C	Control
AgRP	Agouti-related protein
α-MSH	Alpha-melanocyte stimulating hormone
ARC	Arcuate nucleus
BBB	Blood-brain barrier
BMI	Body mass index
CART	Cocaine and amphetammine-related transcript
HPA	Hypothalamus-pituitary-adrenal
LHA	Lateral hypothalamic area
NPY	Neuropeptide Y
NREM	Non-rapid eye movement
POMC	Pro-opiomelanocortin
REM	Rapid-eye movement
sOB-R	Soluble leptin receptor
SR	Sleep restriction
SWS	Slow wave sleep

I. Introduction

Energy homeostasis is a crucial yet delicate process modulated by several systems and hormones. It is defined as the coordination between energy input, mainly food intake, and energy output or expenditure. A recent study conducted by Penev put forward the notion that sleep loss incurs additional metabolic costs, an expenditure previously not emphasized [8]. Therefore, any alteration in sleeping patterns, such as sleep restriction, might disturb the energy balance of the body and, subsequently, the levels of hormones involved in tissue metabolism. Sleep restriction, as defined by the National Sleep Foundation, is less than 7 hours of sleep per night for the average adult. Driven by the demands of daily life, most people find it challenging to sleep for more than 6 hours, making most adults at risk of developing metabolic diseases due to the jeopardized energy homeostatic system. One of the main hormones which convey the status of the body's energy stores is leptin, a 16kDa adipokine mainly secreted by white adipose tissue in response to increased energy intake and which is pleiotropic in nature [9]. Leptin is also referred to as a satiety hormone as it suppresses appetite [2]. A corpus of scientific literature has demonstrated that leptin levels decrease after sleep restriction [8,42]. However, the cause of such dampening of hormone concentration has not been elucidated. Although leptin regulators are many, one main regulator is its own soluble receptor (sOB-R) [25]. sOB-R, a product of the alternative splicing of the diabetes gene-*db*-, was recently identified as the main leptin-binding protein in human blood and is crucial for leptin's biological action [30]. Previous studies have shown that sOB-R followed a significant circadian rhythm inverse to that of leptin [27] and that it modulates the bioavailability of leptin by stabilizing this 16 kDa protein and preventing its clearance from the blood. sOB-R may also bind leptin in order to prevent its attachment to membrane-bound receptors, thereby restricting the initiation of a full cell signaling pathway [7]. Given that sleep restriction affects leptin levels and alters energy homeostasis, the underlying

leptin-mediated mechanisms on tissue metabolism, such as body weight and metabolic syndrome problems, associated with disturbed sleep remain to be elucidated. Therefore, the present study investigated the effect of acute sleep restriction on circulating free leptin and sOB-R levels and their diurnal serum patterns. This is to test the hypothesis that sleep restriction reduces the bioavailability of free leptin at specific times of day via increasing the levels and diurnal expression of its soluble receptor in the blood. This will add to our understanding of the adaptive homeostatic mechanisms that may underlie the body's neuroendocrine response to changes in energy needs incurred by extended wakefulness (i.e. diminished sleep duration). This may also have valuable therapeutic implications, in that it may place sOB-R as a potential drug target in certain metabolic pathways, such as obesity, type 2 diabetes and metabolic syndrome.

II. Literature Review

The Biology of Leptin

Leptin is a key hormone of the body's hunger-satiety system and is; therefore, a mediator of long-term regulation of energy balance [2]. It is released primarily by white adipose tissue and centrally, mainly on the hypothalamus, to inhibit food intake, hence its anorexigenic role [3]. Leptin was discovered in 1994 at Jackson's laboratories after the identification of the *ob* gene in *ob/ob* mice [1]. This preceded a study by Coleman [2] at the same laboratory where obesity in mice was reported in littermates at 4-6 weeks of age. Affected mice exhibited a continuous increase in body weight (would weigh around 4 times more than their normal counterparts), hyperglycemia, and sterility. The offspring of heterozygote mating marked a 3:1 ration, indicative of a recessive gene which was called the *ob* gene. Coleman used parabiosis, a technique which allows animals with different physiologies or genetics to be conjoined and to share their blood supply, to further elucidate the genetic metabolic basis underlying obesity in the Jackson's mice in order to determine the nature of the obese mice found in Jackson Laboratories[3]. Accordingly, he showed that an *ob/ob* mouse joined with a wild type mouse would result in a significant decrease in the appetite and weight of the obese mouse without any adverse effects on the normal mouse. Coleman's study demonstrated that the *ob* mouse was deficient in a "satiety" factor that was part of the metabolic pathways regulating energy balance. This factor or hormone encoded by the *ob* gene was later designated by Friedmann et al. [1] as leptin.

Leptin, a 16 kDa product of the *ob* gene localized on chromosome 7q31, is a 167 amino acid α-helical type 1 cytokine, mainly produced by adipocytes of white adipose tissue [4]. It is also produced, to a lesser extent, by several other organs, such as brown adipose tissue, placenta, ovaries, and stomach. This hormone is an important metabolic signal that has been implicated in

the central and peripheral regulation of energy homeostasis. In humans, circulating leptin concentrations are responsive to acute changes in energy balance resulting from change in caloric intake, fat mass, and sleep duration [8]. Leptin acts on the hypothalamus to convey information regarding the body's energy stores, the central effect of which is suppression of appetite or hunger (thus limiting food intake) [9]. Specifically, the hormone binds to two neuronal populations implicated in energy homeostasis and which are located in the arcuate nucleus (ARC) that, in turn, projects axons to the lateral hypothalamic area (LHA). These ARC cells include the pro-opiomelanocortin(POMC)/cocaine and amphetamine-related transcript (CART) neurons. Leptin binding to these neurons increases the biosynthesis of their respective neurotransmitters which, in turn, generates an anorectic signal through alpha-melanocyte stimulating hormone (α-MSH) [10]. Leptin also binds to the agouti-related protein (AgRP)/neuropeptide Y (NPY) neuronal populations in the hypothalamus to inhibit the expression of the orexigenic signals NPY and AgRP, thus signaling satiety [10].

In addition to its role in the central regulation of the appetite-satiety system, leptin was also found to act on hypothalamic excitatory neuropeptides, referred to as hypocretins or orexins, which have potent wake-promoting effects [11]. The neuroendocrine relationship mediating sleep/wake behavior and leptin secretion was further investigated by Simon et al. [12] who showed that sleep *per se* affected circulating leptin levels; subjects put on enteral nutrition exhibited an increase in nocturnal leptin levels, an increase which was previously attributed to day-time meal ingestion. This pivotal finding triggered other studies in the field to examine the physiological relationship between sleep and energy regulation. This is an intriguing area of research given the fact that the worldwide sleep duration has decreased by one to two hours during the second half of the 20th century [34] and that the incidence of obesity has

concomitantly doubled [14].

Leptin has been also implicated in the endocrine control of peripheral tissue metabolism. It binds to its receptors on pancreatic β-cells to regulate insulin secretion [35], namely through inhibiting it [36]. Insulin regulates leptin, mainly by stimulating leptin's synthesis and secretion. These two hormones also have antagonistic effects: insulin acutely stimulates lipogenesis while decreasing lipolysis, whereas leptin exerts opposite effects. The abnormal accumulation of triglycerides in non-adipose tissues caused by excessive lipogenesis leads to a detrimental state known as lipotoxicity. The latter may be reversed by leptin, making it a potential therapeutic agent for lipodystrophy syndrome [43]. Leptin also acts on skeletal muscles to stimulate free fatty acid oxidation [37]; on adipose tissue to inhibit insulin-mediated glucose uptake, glycogen synthase activity, and lipogenesis[38]; and on the liver to inhibit insulin binding to hepatocytes [39] and glucagon-activated cAMP production [40].

Leptin Receptors Mediate Leptin Action

Leptin acts on its different target tissues using different receptor isoforms. The discovery of leptin receptor also came from studies on a mutant mouse strain and parabiotic experiments [1,2]. In 1966, another mutation occurred in an inbred mouse strain [C57BLKS/J, a widely used mouse model of non-insulin dependent (type 2) diabetes] at Jackson laboratories. In this study, the mice (called *db/db* mice) were diabetic, hyperphagic, fat, and would not survive on a low calorie diet . The results of the parabiotic experiments of the *db/db mice* were contradictory to those of *ob/ob*: when *db/db* mice were parabiosed to a normal mouse, the normal mouse lost a dramatic amount of weight, became hypoglycemic, and died within fifty days of the surgery; whereas, the mutant animal was resistant to the endogenous factor produced by the normal

mouse [2,3]. These experiments suggested that the *ob* gene encodes a soluble factor that circulates in blood, whereas the *db* gene encodes its receptor. Shortly after *ob* sequencing and the designation of its protein product as leptin, the leptin receptor gene in humans was identified using an expression library, and then mapped to the *db* locus localized on chromosome 1p [16]. The leptin receptor is a member of the class I cytokine receptor family [17]. It is expressed in, at least, four variants in human tissue and in, at least, five splice variants (ObRa-e) in mice, all of which share the first 805 amino acids [18]. The longest and most ubiquitous form of these receptors (ObRb) is the only one capable of initiating the full signaling pathways that involve kinase-induced phosphorylation of proteins, including JAK2/STAT3, erbB2, ERK, IRS1 and rho/rac, as it is the only receptor identified in humans that has an intact intracellular domain [19,20]. The shortest form, Obre (or the soluble leptin receptor - sOB-R - as called in humans) is a soluble binding protein that has no transmembrane or cytoplasmic domains and represents the main leptin-binding activity in human blood. This soluble receptor, which circulates in two different N-glycosylated isoforms (as a dimer or in an oligodimerized state) [21], is generated in humans by ectodomain shedding of membrane-bound receptor forms via metalloprotease-mediated cleavage [22]. Thus, no mRNA for a splice form encoding the sOB-R has been detected to-date [23]. Serum leptin binds reversibly to its soluble receptor in a 1:1 ratio [24], which is thought to modulate steady-state leptin levels by forming a complex with free leptin in the circulation, thus preventing hormone degradation and clearance from the blood [25]. Given that high sOB-R levels are present in the blood of lean individuals who [26], due to their decreased amount of adipose tissue, have low levels of leptin, it reinforces the hypothesis that sOB-R may act as a potential reservoir of bioactive leptin.

The Diurnal Blood Profile of Leptin and sOB-R: Relationship with Sleep Duration

Leptin and sOB-R show an out-of-phase circadian variation, with leptin levels being relatively high during the later hours of the day night (as opposed to early hours) [8]. Furthermore, sOB-R is shown to a follow a circadian rhythm identical to that of cortisol which tends to be relatively high during daytime [27]. Sleep restriction studies on human subjects demonstrated an effect of sleep duration on blood leptin levels. In one study, 6 days of sleep restriction 4 hours of sleep resulted in an average decrease of leptin levels and daily rhythm amplitude by around 20% , and a concomitant increase in cortisol levels [8]. The decrease of free leptin following sleep restriction has been widely described in the literature, however the exact physiological mechanisms by which sleep duration affects leptin levels and, hence, cellular metabolism, remain unclear.

Sleep duration appears to affect the concentration of free leptin via several mechanisms. One pathway employs the autonomic nervous system whereby increased sympathetic nerve activity appears to mediate the effect of short sleep duration on decreased leptin levels [28]. This proposed neural mechanism; however, is controversial since other related studies failed to confirm the concept of regulatory feedback inhibition of the hormone's release by the sympathetic nervous system [29]. Another plausible mechanism considers the interaction between sleep duration and the hypothalamus-pituitary-adrenal (HPA) axis activity as to affect leptin release. This is manifested by the increased secretion of cortisol following sleep restriction, which reflects an increased activity of the HPA-axis in response to stress (resulting from short sleep duration) [41]. The endocrine changes (such as increased cortisol and decreased

leptin levels) that accompany the stress response have an important adaptive purpose: they favor metabolic pathways that provide extra energy supplies to body tissues in anticipation of a fight-or-flight response. It is well-documented that the HPA axis is leptin-sensitive; leptin appears to act on the hypothalamus to inhibit cortisol release [31,32,33], despite findings on the effect of cortisol on leptin secretion have not been consistent in the literature.

Many neuroendocrine pathways appear to be involved in leptin regulation which is quite intricate and complex. Among these, the soluble leptin receptor (sOB-R) is the main regulator of leptin levels and this regulation is bidirectional. An increase in blood leptin levels is accompanies by a decrease in sOB-R and vice versa [33]. The increase in sOB-R that accompanies decreased leptin levels serves to 1) delay the clearance of leptin from the circulation since unbound (free) leptin is subject to quick degradation in the blood and 2) limit the transport of unbound leptin into the brain tissue through the blood-brain barrier (BBB) [25]. These safeguarding mechanisms help prevent the adverse physiological effects that may ensue as a result of low leptin levels knowing that leptin is involved in the regulationand modulation of several biological processes. These include energy homeostasis [34], angiogenesis [35], bone growth [36], cardiovascular function [37], and inflammatory response [38].

Despite the growing number of studies that have investigated the biological mechanisms that mediate the effects of short sleep duration on leptin release, none has looked at a potential accompanying change in sOB-R blood profile. Based on the knowledge that leptin regulates its own soluble receptor as to maintain energy homeostasis, the aim of the present study was to investigate the effect of acute sleep restriction on the diurnal variation of sOB-R blood levels and

to test the hypothesis that acute sleep restriction causes curtailment of blood leptin via altering the diurnal rhythm (the phase and/or amplitude) of its soluble receptor - sOB-R-. Understanding the impact of short sleep duration on the sOB-R levels may help elucidate, in part, the physiological mechanisms by which sleep restriction alters energy homeostasis and mediates the associated body weight and metabolic problems that emanate from insufficient sleep.

III. Materials and Methods

Subjects

Five healthy females gave informed consent to participate in this study, approved by the Institutional Research Board at Notre Dame University-Louaize. All participants were recruited from the Zouk Mikael area and met the following inclusion criteria: female; 20–30 years of age; non-smoker; non-alcoholic drinker; exhibited habitual daytime activity (i.e., no shift-work); had not travelled over 3 or more time zones within 6 weeks prior to the study; had habitual sleep duration of at least 7–8 h/night and no frequent daytime naps; had no history of health disorders (e.g. eating, sleep, metabolic, endocrine, reproductive disorders, or any other health/lifestyle condition that is known to affect sleep patterns), and had no ongoing hormone treatments. Professional and semi-professional athletes were also excluded due to potential disruptions in menstrual cycles. All participants did not suffer from obesity and were all within the BMI range 22-26. Participants kept a food and sleep diary.

Experimental Protocol

Subjects were maintained on an 8-hour sleep/wake schedule (bed-wake times 12:00 AM - 8:00 AM, EST) for one week prior to the experimental period. This week constituted the baseline [control (C)] period before subjecting participants to 2 nights of restricted sleep [experimental period (SR), bed-wake times 3:30 AM – 8:00 AM, EST]. Throughout the duration of the study, all subjects followed a weight-maintaining diet consumed at the following average time points (± 30 min): breakfast, 9:00 AM; lunch, 3:00 PM; and dinner, 7:00 PM.

Blood and Saliva Sampling

Timed blood and saliva samples were collected from each subject on the days before and following the 2 nights of sleep restriction [control or baseline (C) vs. sleep restriction (SR),

respectively). Fluid sampling occurred at the following time points: 8:00 AM, 12:00 PM, 4:00 PM, 8:00 PM, and 12:00 AM. Blood was drawn using a medium-flow BD Microtainer Contact-activated Lancet(Microtainer Cat#366593) and drops were collected in 0.5 ml K2E K2EDTA tubes (Minicollect Item # 450532), centrifuged at 1000g for 15 minutes and stored at -20° C for later analysis of leptin and soluble leptin receptor (sOB-R) blood concentrations. Saliva samples were collected upon awakening using the passive drooling technique and stored at -20° C for later analysis of cortisol levels.

Sleep Recording and Body Fluid Analysis

Sleep was polygraphically recorded during the baseline night and the 2 nights of SR using the Fitbit Charge 2. The recordings were scored at 20-sec intervals in stages wake, I, II, III, IV, and REM (rapid eye movement) according to standard criteria. Sleep onset and final awakening were defined as the time corresponding to the first and last 20-sec interval scored II, III, IV, or REM. The following parameters were determined: sleep period (*i.e.* time interval separating sleep onset from morning awakening), total sleep time (*i.e.* the total sleep episode less the awake time), sleep efficiency (*i.e.* total sleep time/time spent in bed), duration of light non-rapid eye movement (NREM) sleep (*i.e.* stages 1 and2), duration of slow wave sleep (SWS) (*i.e.* sleep stages III and IV), duration of REM sleep, and duration of intra-sleep wakefulness.

Salivary cortisol levels were measured using electrochemiluminescence immunoassay (La Roche Cobas® 6000). The blood levels of free leptin and its soluble receptor (sOB-R) were measured using specific enzyme-linked immunoassay kits (ELISA) (R&D Quantikine® ELISA Cat. # DLP00 and Cat. # DOBR00, respectively). Soluble leptin receptor (sOB-R) levels were measured using ELISA kit (R&D Quantikine® ELISA)

To minimize assay variability, all samples collected from different subjects were assayed at the same time.

Statistical Analysis

All statistical analysis were run on SPSS Statistics Version 25.0.

Repeated measure one-way ANOVA with pairwise comparison was used for multiple comparisons within a group (i.e. to compare hormone or soluble receptor concentrations at different time points within a group - C or SR); paired *t*-test was used for paired comparisons between groups (i.e. to compare hormone or soluble receptor concentration at a given time point between C and SR). In both comparisons, a p-value <0.05 was considered significant.

Results are presented as mean ± SEM.

III. Results

1. Sleep parameters

Sleep efficiency significantly increased by 4.2% following 2 nights of sleep restriction compared to control (92.09 % vs. 88.39 %, respectively; $p < 0.01$). Significant differences in the sleep stages were also observed: subjects spent lesser time in both the light and deep stages of NREM sleep in the SR than the C periods (light sleep: 2.43 ± 0.04 hrs vs. 4.43 ± 0.03 hrs, respectively; $P < 0.001$; deep sleep: 0.83 ± 0.02 hrs vs. 1.33 ± 1.03 hrs, respectively; $P < 0.01$). Similarly, the time spent in REM sleep was shorter following SR than the C periods (0.99 ± 0.02 hrs vs. 1.42 ± 0.05 hrs, respectively; $P < 0.01$). Table 1 summarizes the different sleep parameters which were measured before and after acute sleep restriction.

Table 1. Sleep parameters in subjects during the control period and following two nights of sleep restriction.

	C	SR	P-value
Time in bed	8h 26mn ± 3.5mn	4h 38mn ± 2.5mn	**<0.01**
Time awake	58.75mn ±2.7mn	22mn ±1.21mn	**<0.01**
REM	1h 25mn ±2.85mn	59.25mn ±0.92mn	**<0.01**
Light Sleep	4h 26mn ±1.75mn	2h 25.6mn ±2.13mn	**<0.01**
Deep Sleep	1h 20mn ±1.85mn	49.75mn ±1.56mn	**<0. 01**
Sleep Duration	7h 27mn ±2.25mn	4h 15mn ±1.34mn	**<0.01**
Sleep efficiency (SE)	88.39%	92.09%	**<0.01**

Results represent mean ± SEM. Comparisons between groups are based on paired t-test.
C, control or baseline; SR, sleep restriction.

2. The effect of acute sleep restriction on serum free leptin levels.

The average diurnal serum leptin concentration in C was 25.71 ± 1.71 ng/ml. Hormone concentration varied across the times of day, fluctuating every 4 hours, and there appeared to be significant differences between the different time points (one -way repeated measures ANOVA: $F(4, 16) = 4.063$, $P < 0.05$). Based on post hoc analysis, hormone levels were significantly higher early in the morning as opposed to later times of day (refer to table 2). Restricting nocturnal

sleep to 4 hours over 2 nights did not alter the diurnal 'baseline' pattern of the hormone, but rather blunted it. That is, the average diurnal serum leptin levels were significantly lower in SR than C (20.94 $\pm$ 1.71 ng/ml vs. 25.71 $\pm$ 1.71 ng/ml, respectively; $P < 0.01$). In SR, there were highly significant differences in hormone concentration at different times of day (one-way repeated measures ANOVA: $F(4, 16) = 10.03$, $P < 0.01$), with circulating free leptin being significantly lower in the late evening (refer to table 2).

Except for 8:00 AM, the mean free leptin concentrations were higher in C than SR at all measured time points: 12:00 PM (23.18 $\pm$ 6.52 ng/ml vs. 17.28 $\pm$ 4.32 ng/ml, respectively; $P < 0.05$), 4:00 PM (25.78 $\pm$ 6.69 ng/ml vs. 19.67 $\pm$ 4.48 ng/ml respectively, $P<0.05$), 8:00 PM(21.56 $\pm$ 4.4 vs. 18.76 $\pm$ 3.98 ng/ml, respectively; $P<0.01$), and 12:00 AM (28.23 $\pm$ 5.62 ng/ml vs. 23.48 $\pm$ 5.1 ng/ml, respectively; $P<0.01$). Figure 1 summarizes the serum levels of free leptin measured at 4-hour intervals, starting 8:00AM, before and after sleep restriction.

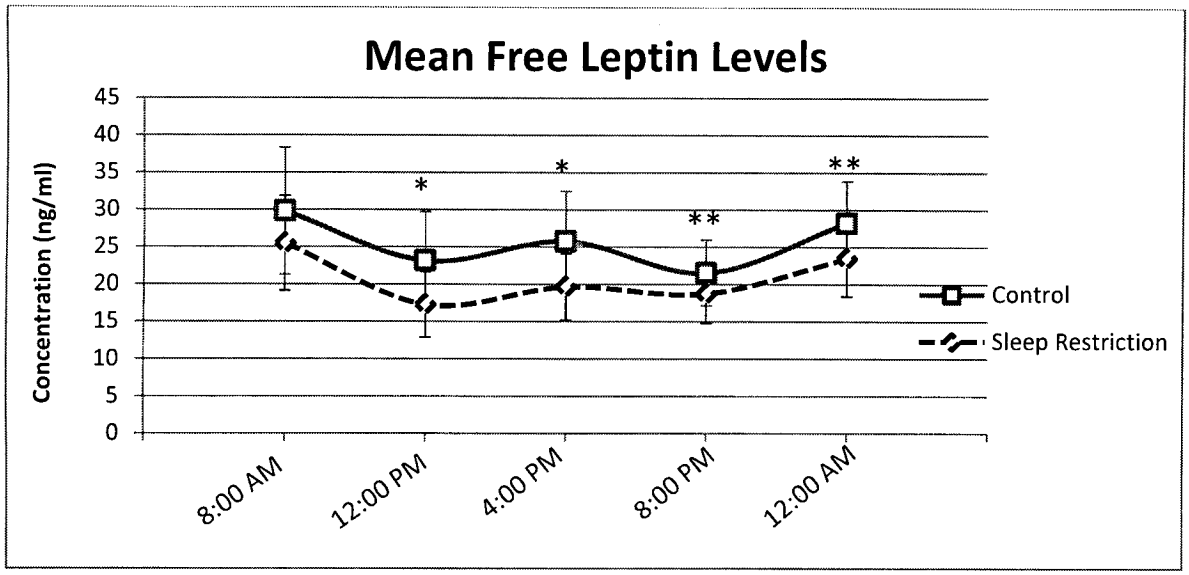


Figure 1. The effect of acute sleep restriction on the mean serum levels of free leptin.
Subjects who maintained an 8-hour bed schedule (bedtime at 12:00 AM) had relatively higher levels of free leptin at any measured time point during the day than after SR (bedtime at 3:30 AM). Significant differences were apparent at all times except wake time (8:00 AM). Two nights of sleep restriction did not appear to alter the normal daily pattern of serum leptin concentration. *Error bars* represent the SEM of the average value. *P<0.05 and **P<0.001 denote significant and highly significant differences, respectively, based on paired t-test comparisons between groups.

Table 2. Daily variation of circulating free leptin before (control) and following sleep restriction

	Time	Free Leptin Concentration (ng/ml)
Control	8:00 a.m.	29.8 ± 8.54
	12:00 p.m.	25.75 ±6.52 [a]
	4:00 p.m.	25.78 ± 6.69 [a,b]
	8:00 p.m.	21.56 ± 4.4
	12:00 a.m.	28.23 ± 5.62 [d]
SR	8:00 a.m.	25.5 ± 6.36
	12:00 p.m.	17.28 ± 4.39 [a]
	4:00 p.m.	19.67 ± 4.48 [a,b]
	8:00 p.m.	18.75 ± 3.98 [a]
	12:00 a.m.	23.48 ± 5.1 [b,d]

The reported values represent mean free leptin concentration (measured at a specific time point) ± SEM. There was a significant difference in hormone levels across the different times of day: [a] $p < 0.05$ analyzed using one-way repeated measures ANOVA post hoc analysis (95% confidence interval) for comparison with hormone concentration measured at 8:00 AM; [b] $p < 0.05$ analyzed using ANOVA pairwise comparisons (95% confidence interval) for comparison with hormone concentration measured at 12:00 PM; [d] $p < 0.05$ analyzed using ANOVA pairwise comparisons (95% confidence interval) for comparison with hormone concentration measured at 8:00 PM.

3. The effect of acute sleep restriction on the soluble leptin receptor (sOB-R) serum levels.

The average diurnal serum sOB-R concentration in C was 19.79 + 1.643 ng/ml. Receptor serum levels fluctuated at 4-hours intervals, and there appeared to be significant differences in sOB-R concentration at different time points (one-way repeated measures ANOVA: $F(4,16) = 6.622$, $P < 0.01$). Based on post hoc analysis, the soluble receptor levels were significantly elevated in the late evening (refer to table 3). Restricting nocturnal sleep to 4 hours over 2 nights resulted in a highly significant increase in the average serum sOB-R levels when compared to C (24.39 $\pm$ 1.21 ng/ml vs. 19.79 $\pm$ 1.643 ng/ml, respectively; $P < 0.01$), but no change in the diurnal 'baseline'

pattern. The average receptor concentration varied across the times of day and there appeared to be significant differences between the different time points (one-way repeated ANOVA: $F(4, 16) = 6.746$, $P < 0.01$). Results are graphically represented in figure 2.

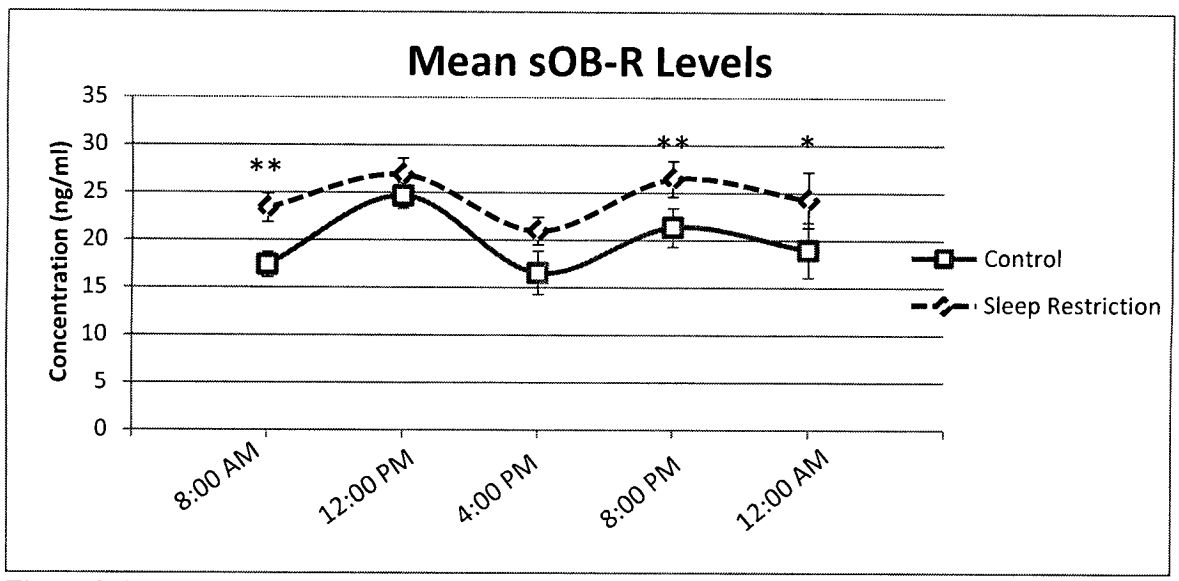


Figure 2. The effect of acute sleep restriction on soluble leptin receptor (sOB-R) levels. Restricting sleep to an average of 4 hours a night for 2 consecutive nights resulted in higher diurnal sOB-R levels compared to baseline (control) measurements. Significant differences in receptor levels were observed at 8:00 AM, 8:00 PM, and 12:00 AM. Sleep restriction; however, did not alter the normal daily pattern of sOB-R levels in serum. *P<0.05 and **P<0.001 denote significant and highly significant differences, respectively, based on paired t-test comparisons between groups; *error bars* represent the SEM of the average value.

Significant time point differences in average sOB-R between pre- and post-SR conditions were observed: serum receptor levels were significantly lower in C than SR at 8:00 AM (17.38 $\pm$ 1.32 ng/ml vs. 23.37 $\pm$ 1.5 ng/ml, respectively; P<0.01), 8:00 PM (21.36 $\pm$ 2.03 ng/ml vs. 26.46 $\pm$ 1.86 ng/ml, respectively; P<0.01), and 12:00 AM (18.98 $\pm$ 2.92 ng/ml vs. 24.27 $\pm$ 2.93 ng/ml, respectively; P<0.05)

Table 3 . Daily variation of circulating sOB-R before (control) and following sleep restriction

	Time	s-OR Concentration (ng/ml)
Control	8:00 a.m.	17.38 ± 1.32
	12:00 p.m.	24.67 ± 1.34 [a]
	4:00 p.m.	16.55 ± 2.26 [b]
	8:00 p.m.	21.36 ± 2.03 [a,b]
	12:00 a.m.	18.97 ± 2.92 [b]
SR	8:00 a.m.	23.37 ± 1.5
	12:00 p.m.	27.13 ± 1.71
	4:00 p.m.	20.97 ± 1.45 [b]
	8:00 p.m.	26.46 ± 1.86 [a,c]
	12:00 a.m.	24.27± 2.93

The reported values represent mean free leptin concentration (measured at a specific time point) ± SEM. There was a significant difference in receptor levels across the different times of day: [a] $p < 0.05$ analyzed using one-way repeated measures ANOVA post hoc analysis (95% confidence interval) for comparison with receptor concentration measured at 8:00 AM; [b] $p < 0.05$ analyzed using ANOVA pairwise comparisons (95% confidence interval) for comparison with receptor concentration measured at 12:00 PM; [d] $p < 0.05$ analyzed using ANOVA pairwise comparisons (95% confidence interval) for comparison with receptor concentration measured at 8:00 PM.

4. sOB-R and free leptin diurnal patterns of expression in serum.

Comparison of the diurnal serum patterns of leptin and sOB-R in either C or SR demonstrated an out-of-phase variation at the measured time points. Figure 3.A shows that the serum concentration of free leptin hormone in C was higher than that of its receptor, which may reflect their respective molarities. In contrast, the serum concentration of sOB-R was higher than that of free leptin following SR (figure 3.B). Sleep restriction minimized the normal concentration differences between free leptin and sOB-R depicted at 8:00 AM, 4:00 PM, and 12:00 AM, yet, unlike control, widened the concentration gap at 12:00 PM and 8:00 PM.

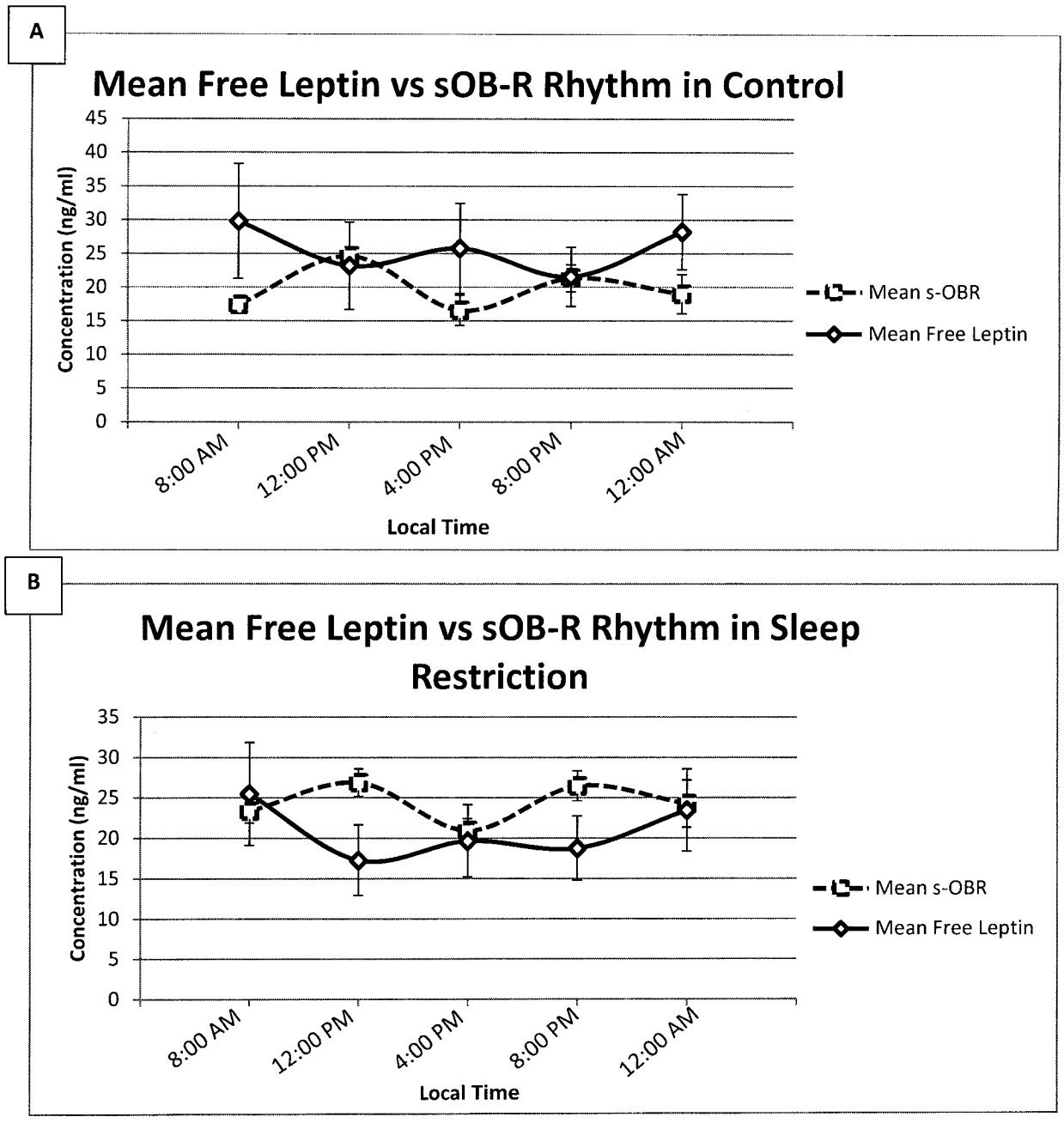


Figure 3. The daily variation in serum levels of free leptin and sOB-R before (A) and following 2 nights of sleep restriction (B). Sleep restriction minimized the normal concentration differences between free leptin and sOB-R depicted at 8:00 AM, 4:00 PM, and 12:00 AM in control, yet, unlike control, widened the concentration gap at 12:00 PM and 8:00 PM. *Error bars* represent the SEM of the average value.

5. Cortisol measurement

The concentration of salivary cortisol, a commonly used marker for stress, was measured at 8:00AM in order to determine the impact of sleep restriction on the hypothalamo-pitutitary-adrenal axis (HPA axis). After 2 nights of sleep restriction, the mean salivary cortisol concentration significantly increased from baseline conditions (8.01 ± 1.6 nmol/L vs. 6.21 ± 0.72 nmol/L, respectively; $P<0.05$).

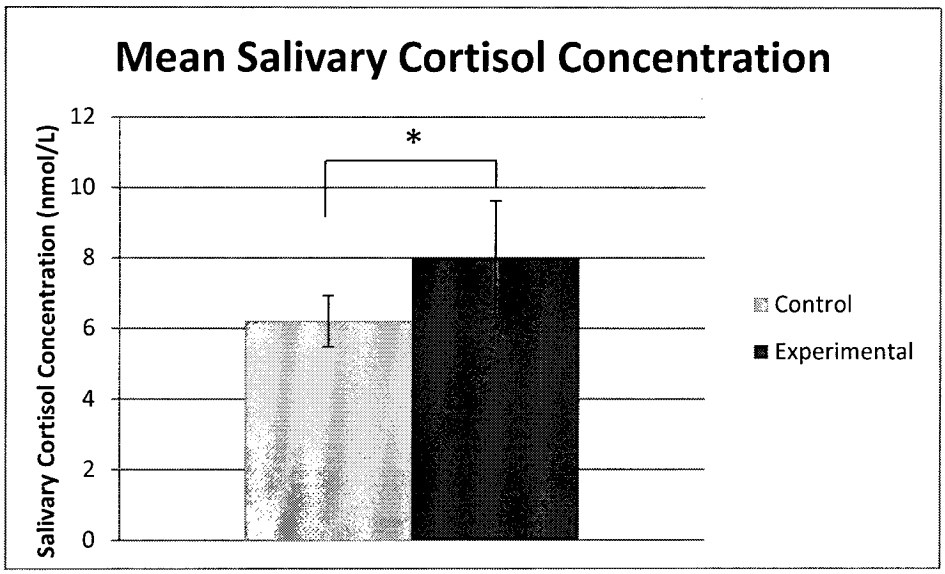


Figure 4. Mean salivary cortisol levels for control and sleep restriction periods. The average concentration of salivary cortisol, collected at 8:00 AM, was significantly higher after 2 night of sleep restriction relative to control (baseline). * indicates statistical significance at $P<0.05$ based on paired t-test. *Error bars* represent the SEM of the average value.

IV. Discussion

To our knowledge, the present study is the first to describe the diurnal variation in circulating leptin receptor levels (sOB-R) following acute sleep restriction in healthy females. Our results showed that restricting nocturnal sleep to 4 hours a night for two consecutive nights affected the average daily levels, but not the normal diurnal pattern, of sOB-R. SR resulted in a 23.24% increase of average serum sOB-R relative to baseline. Significant differences in serum sOB-R between SR and baseline were observed at specific times of the day, namely early in the morning (at 8:00 AM) and during the first half of the night (at 8:00 PM and 12:00 PM). These observations add new knowledge to our understanding of the physiological mechanisms by which partial sleep deprivation interacts with the endocrine system that regulates energy homeostasis as to affect body metabolism.

Leptin, an adipokine synthesized by adipose tissue in response to feeding, is released into the systemic circulation in a circadian pattern, with peak levels in the late evening and early morning and nadir in the late morning [33]. This diurnal variation is consistent with our observation that circulating free leptin levels were significantly high in the early morning, around mid-afternoon, and at mid-night. Circadian regulation of leptin release ensures that, in concert with other metabolism-regulating hormones, food intake is consolidated to the daily sleep/wake cycle such that feeding occurs at times of day when metabolic demands are optimal. Energy homeostasis has been shown to be adversely affected by partial sleep deprivation in humans, one mechanism involving the leptin signaling pathway (both central and peripheral pathways) [9,10,29,35]. Based on previous studies, sleep restriction caused a decrease in serum free leptin levels [8]. Similarly, we observed a blunting of the free hormone levels following 2 nights of sleep restriction, where the mean serum free leptin concentration decreased by 18.56% as compared to

mean baseline values. The daily pattern of free leptin; however, was unaltered following acute sleep restriction.

Besides being under circadian control, total circulating leptin levels has been shown to be regulated by its soluble receptor levels (sOB-R) [30]. SOB-R, a transcript of the diabetes (*db*) gene [33], delays leptin clearance from the blood and is an important determinant of free (unbound) leptin levels, which is the biologically active form the hormone [6, 25]. Therefore, we further looked at sOB-R levels and diurnal, expression following SR to gain insight into the physiological mechanisms by which nocturnal sleep duration affects body metabolism. Our results showed that the average daily levels of sOB-R increased by 23.24% relative to baseline. This may explain the observed concomitant drop in the average daily free leptin levels following SR, which implies that more of the circulating hormone might be present in the bound, rather than the free, form. It is intriguing to speculate that delaying bedtime by 3.5 hours (and thus restricting nocturnal sleep to 4.5 hours a night) resulted in up-regulating sOB-R, probably by leptin-mediated mechanisms, for the purpose of lowering free leptin levels and making it less available to tissues. This my implicate leptin's biological role in regulating feeding behavior and energy homeostasis; prolonged wakefulness (such as delaying bedtime) may require less circulating free leptin in order to meet the body's metabolic or energy demands imparted by the waking state.

We also observed diurnal variation of sOB-R levels, with fluctuations depicted at 4-hour intervals. It is well documented that levels of sOB-R follow a circadian rhythm that is discordant to that of circulating leptin [27]. In our study, we did not expand the time window of sOB-R measurement as to delineate its circadian rhythmicity, but we rather looked at the consequences of SR on the sOB-R daily pattern. In this respect, our results showed that SR did not alter the

diurnal sOB-R pattern. As stated previously, the effect of SR was mainly on the levels of the soluble receptor which were significantly elevated at midday 12:00 PM) and in the late evening/early night (8:00 PM).

In order to further understand the physiological relationship between sOB-R and serum free leptin in SR, we compared their diurnal variation before and after restricted sleep. Based on the present results, the free form of the hormone and its soluble receptor exhibited an inverse (discordant) diurnal pattern with respect to each other in both the normal and restricted sleep states. This was evident at all measured time points. These observations are in agreement with other reports on human subjects which demonstrated an out-of-phase relationship between leptin and its soluble receptor in normal feeding and fasting states (33). This inverse circadian relationship is also shown to be conserved when considering other biological determinants such as obesity, glycemic state, and sex of the individual [5]. The novel finding of our study is that SR attenuates free leptin levels by presumably altering the levels, rather than the diurnal pattern, of sOB-R. Prior to sleep restriction (i.e. baseline period), the concentration of free leptin was higher than that of sOB-R. In contrast, SR resulted in relatively higher sOB-R than free leptin, the difference being significant at midday and early in the night. Taken together, our results may lend support to other reports suggestive of a regulative role of leptin on its own soluble receptor levels [25]. That is, behavioral states characterized by relatively high energy demands (such as prolonged wakefulness, fasting) are associated with relatively lower circulating leptin levels and higher sOB-R. As a physiological adaptation, leptin upregulates its own soluble receptor (thus decreasing the circulating free form of the hormone) to maintain energy homeostasis.

In order to ascertain that the reduction of sleep hours impacted the subjects' homeostatic physiological status, we examined fasting levels of cortisol, a commonly used stress-marker. Measurements of salivary cortisol demonstrated an increase by about 29% in the hormone's concentration following SR. This may suggest that restricted sleep is accompanied by activation of the HPA axis since sleep insufficiency imposed physiological stress. This observation may also support other findings which showed an intricate relationship between cortisol and leptin levels [5]. Leptin can blunt the stress-induced activity of the HPA-axis and; therefore, a decrease in serum leptin concentration would not provide the adequate feedback inhibition to suppress the extended release of cortisol from the adrenal medulla. The interplay between leptin and cortisol facilitates the integration of peripheral information about energy storage with centrally processed information about stress-coping strategies. This implies that the decrease of leptin levels following just two nights of sleep restriction has serious implications on both metabolic and mental health [35].

V. Conclusion

Given that sleep curtailment is becoming increasingly prevalent in modern society, the results of this study present an additional dimension not previously explored with regards to the importance of adequate sleep quality and quantity. Based on our findings, SR appeared to lower free leptin levels by upregulating sOB-R rather than altering its diurnal pattern. Though our study did not investigate the cause-effect relationship between leptin levels and sOB-R, our results may support the notion that, following SR, leptin mediates the overexpression of its own soluble receptor as an adaptive physiological response to tissue metabolic needs. Considering leptin's role in energy balance, this may explain altered feeding behaviors, body weight problems, and increased susceptibility to metabolic syndrome associated with insufficient sleep [2, 9, 27].

The biological action of leptin is controlled by its soluble receptor depending on certain metabolic or environmental conditions. This study puts forward the notion that the decrease in bioactive leptin following acute sleep restriction in healthy females, may be partly due to the increase of its soluble leptin receptor. In the future, regulatory pathways controlling the sOB-R release into the circulation, as well as its binding capacity to leptin, require further elucidation.

VI. References

1. Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold and J.M. Friedman, Positional cloning of the mouse *obese* gene and its human homolog. *Nature* 372:425-432 (1994).
2. D.L. Coleman, Diabetes-obesity syndromes in mice. *Diabetes* 32, Suppl. 1:1-6 (1982)
3. D.L. Coleman, Effects of parabiosis of obese with diabetic and normal mice. *Diabetologia* 9, 294-298 (1973).
4. Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK et al. Crystal structure of the obese protein leptin-E100. Nature 387 206–209.(1997)
5. Sinha, M. K., Ohannesian, J. P., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Magosin, S.,Caro, J. F. (1996). Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *Journal of Clinical Investigation,97*(5), 1344-1347. doi:10.1172/jci118551
6. Landt, M. (2000). Leptin binding and binding capacity in serum. *Clin Chem,46*(3), 379-384.
7. Holm, J., Gamborg, M., Ward, L. C., Gammeltoft, S., Kaas-Ibsen, K., Heitmann, B. L., & Sørensen, T. I. (2011). Tracking of Leptin, Soluble Leptin Receptor, and the Free Leptin Index during Weight Loss and Regain in Children. *Obesity Facts,4*(6), 461-468. doi:10.1159/000335121
8. K. Speigel, R. Leproult, M. Hermite-Baleriaux, G. Copinschi, P.D. Penev and E. Van Cauter, Leptin levels are dependent on Sleep Duration: Relationships with sympathovagal balance, carbohydrate regulation, cortisol and thyrotropin. *The Jounal of Clinical Endocrinology & Metabolism* 89(11): 5762-5771 (2004)
9. Elmquist JK, Coppari R, Balthasar N, Ichinose M, Lowell BB: Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. J Comp Neurol; 493: 63–71 (2005)
10. Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbaek C, Flier JS, Saper CB, Elmquist JK: Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron; 23: 775–786. (1999)
11. Sutcliffe JG, de Lecea L 2000 The hypocretins: excitatory neuromodulatory peptides for multiple homeostatic systems, including sleep and feeding. J NeurosciRes 62:161–168
12. Simon C, Gronfier C, Schlienger JL, Brandenberger G 1998 Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition:relationship to sleep and body temperature. J Clin Endocrinol Metab 83:1893–1899
13. National Sleep Foundation 2001 "Sleep in America" poll. Washington DC: National Sleep Foundation; 1–113
14. Flegal KM, Carroll MD, Ogden CL, Johnson CL 2002 Prevalence and trends in obesity among US adults, 1999–2000. JAMA 288:1723–1727
15. K.P. Hummel, M.M. Dickie, and D.L. Coleman, Diabetes, a new mutation in the mouse. Science 153, 1127-1128(1966).
16. L. A. Tartaglia, M. Dembski, X. Weng, N. Deng, J. Culpepper, R, Devos, G. J. Richards, L. A. Campfield, F. T. Clark, J. Deeds, C. Muir, S. Sanker, A. Moriarty, K. J. Moore, J. S. Smutko, G. G. Mays, E. A. Wool, C, A. Monroe, and R, 1. Tepper,Identification and expression cloning of a leptin receptor, OB-R. *Cell,* 83, 1263-71 (1995).

17. Bazan JF 1990 Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934–6938
18. J. A. Cioffi, A. W. Shafer, T. J. Zupancic, J. Smith-Gbur, A. Mikhail, D. Platika, and H. R. Snodgrass, Novel B2I9/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat Med,* 2, 585-9 (1996)
19. N. Ghilardi, S. Ziegler, A. Wiestner, R. Stoffel, M. H. Heim, and R. C. Skoda, Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad* 5c(t /5 A, 93, 6231-5(1996).
20. M. G. Myers, Jr., Leptin receptor signaling and the regulation of mammalian physiology, *Rec Prog Horn Res,* 59, 287-304 (2004).
21. Lammert A, KiessW, Glasow A, Kratzsch J. Soluble leptin receptor represents the main leptin binding activity in human blood. Biochem Biophys Res Commun 2001; 283: 982–988
22. Chua Jr SC, Koutras IK, Han L, Liu SM, Kay J, Young SJ, Chung WK, Leibel RL. Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts. Genomics 1997; 45: 264–270.
23. Maamra M, Bidlingmaier M, Postel-Vinay MC, Wu Z, Strasburger CJ, Ross RJM. Generation of human soluble leptin receptor by proteolytic cleavage of membrane-anchored receptors. Endocrinology 2001; 142: 4389–4393.
24. Devos R, Guisez Y, Van der Heyden J, White DW, Kalai M, Fountoulakis M, Plaetinck G. Ligand-independent dimerizationof the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. J Biol Chem 1997; 272: 18304–18310
25. Huang L, Wang Z, Li C. Modulation of circulating leptin levels by its soluble receptor. J Biol Chem 1995; 276: 6343–6349.
26. Laimer M, Ebenbichler CF, Kaser S, Sandhofer A,Weiss H, Nehoda H, Aigner F, Patsch JR. Weight loss increases soluble leptin receptor levels and the soluble receptor bound fraction of leptin. Obes Res 2001; 10: 597–601.
27. Gavrila, A., Peng, C., Chan, J. L., Mietus, J. E., Goldberger, A. L., & Mantzoros, C. S. (2003). Diurnal and Ultradian Dynamics of Serum Adiponectin in Healthy Men: Comparison with Leptin, Circulating Soluble Leptin Receptor, and Cortisol Patterns. The Journal of Clinical Endocrinology & Metabolism, 88(6), 2838-2843. doi:10.1210/jc.2002-021721
28. Akerstedt T, Froberg JE 1979 Sleep and stressor exposure in relation to circadian rhythms in catecholamine excretion. Biol Psychol 8:69–80
29. N. Eikelis, M. Schlaich, A. Aggarwal, D. Kaye, M. Esler, Interactions between leptin and the human sympathetic nervous system
30. Zastrow, O., Seidel, B., Kiess, W., Thiery, J., Keller, E., Böttner, A., & Kratzsch, J. (2003). The soluble leptin receptor is crucial for leptin action: Evidence from clinical and experimental data. *International Journal of Obesity,27*(12), 1472-1478. doi:10.1038/sj.ijo.0802432
31. Wauters M, Considine RV, Van Gaal LF 2000 Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 143:293–311
32. Casanueva FF, Dieguez C 1999 Neuroendocrine regulation and actions of leptin. Front Neuroendocrinol 20:317–363

33. J.Chan, S. Bluher, N. Yiannakouris, M. Suchard, J. Kratsch and C. Mantzoros. Regulation of Circulating Soluble Receptor Levels By Gender, Adiposity, Sex Steroids and Leptin . *Diabetes Vol 51* 2002
34. L.A. Campfield, F.J. Smith, Y. Guisez, R. Devos, P. Burn, Recombinant mouse OB protein: evidence for a peripheralsignal linking adiposity and central neural networks, science 269 (1995) 546–549.
35. Guillemnault,C., Driver H.S., Morin, C. M., & Allen, R. (2011). Welcome Address. Sleep Journal, 12(Suppl.), Vi-Vii. Doi:https://doi.org/10.1016/S1389-9457(11)70001-Kieffer TJ, Heller RS, Habaner JF. Leptin receptors expressed on pancreatic b-cells *Biochem Biophys Res Commun* 1996 **224**: 522–527.
36. Seufert J, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C, Habener JF. Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus *J Clin Endocrinol Metab* 1999 **84**: 670–676.
37. Solini A, Bonora E, Bonadonna R, Castellino P, DeFronzo RA. Protein metabolism in human obesity: relationship with glucose and lipid metabolism and with visceral adipose tissue *J Clin Endocrinol Metab* 1997 **82**: 2552–2558
38. Muller G, Ertl J, Gerl M, Preibisch G. Leptin impairs metabolic actions of insulin on isolated rat adipocytes *J Biol Chem* 1997 **272**: 10585–10593.
39. Nowak K, Mackowiak P, Nogowski L, Szkudelski T, Malendowicz. Acute action on insulin blood level and liver insulin receptor in the rat *Life Sci* 1998 **63**: 1347–1352.
40. Zhao AZ, Shinohara MM, Huang D, Schimizu M, Eldar-Finkelman H, Krebs EG, Beavo JA, Bornfeldt KE. Leptin induces insulin-like signaling that antagonises cAMP elevation by glucagon in hepatocytes *J Biol Chem* 2000 **275**: 11348–11354
41. Meerlo P., Sgoifo A., Suchecki D. Restricted and disrupted sleep: effects on autonomic function, neuroendocrine stress systems and stress responsivity. Sleep Med Rev. 2008;12(3):197–210.
42. Omisade, A., Buxton, O. M., & Rusak, B. (2010). Impact of acute sleep restriction on cortisol and leptin levels in young women. Physiology & Behavior, 99(5), 651-656. doi:10.1016/j.physbeh.2010.01.028
43. Paz-Filho, G., Wong, M., Licinio, J., & Mastronardi, C. (2012). Leptin therapy, insulin sensitivity, and glucose homeostasis. *Indian Journal of Endocrinology and Metabolism,16*(9), 549. doi:10.4103/2230-8210.105571