Original Research *Highlight article*

Visceral fat sympathectomy ameliorates systemic and local stress response related to chronic sleep restriction

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Impact Statement

The present work provides evidence that the visceral adipose tissue sympathetic pathway may modulate the stress response related to sleep restriction locally and systemically. We demonstrated that visceral white adipose tissue (WAT) denervation ameliorated corticosterone serum levels and 11β-HSD1 activity that modified glucocorticoid levels in the adipose tissue of animals submitted to sleep restriction. In addition, visceral WAT sympathectomy decreased adiposity and weight gain, improved glucose tolerance, and reduced daily body temperature profile in sleep-restricted animals. These results suggest that disturbed adipose-brain communication might be part of the metabolic and physiological alterations provoked by shortening sleep time.

Abstract

Disturbance of sleep homeostasis encompasses health issues, including metabolic disorders like obesity, diabetes, and augmented stress vulnerability. Sleep and stress interact bidirectionally to influence the central nervous system and metabolism. Murine models demonstrate that decreased sleep time is associated with an increased systemic stress response, characterized by endocrinal imbalance, including the elevated activity of hypothalamic–pituitary–adrenal axis, augmented insulin, and reduced adiponectin, affecting peripheral organs physiology, mainly the white adipose tissue (WAT). Within peripheral organs, a local stress response can also be activated by promoting the formation of corticosterone. This local amplifying glucocorticoid signaling is favored through the activation of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). In WAT, 11β-HSD1 activity is upregulated by the sympathetic nervous system, suggesting a link between sleep loss, augmented stress response, and a potential WAT metabolic disturbance. To gain more understanding about this relationship, metabolic and stress responses of WAT-sympathectomized rats were analyzed to identify the contribution of the autonomic nervous system to stress response-related metabolic disorders during chronic sleep restriction. Male Wistar rats under sleep restriction were allowed just 6 h of daily sleep over eight weeks. Results showed that rats under sleep restriction presented higher serum corticosterone, increased adipose tissue 11β-HSD1 activity, weight loss, decreased visceral fat, augmented adiponectin, lower leptin levels, glucose tolerance impairment, and mildly decreased daily body temperature. In contrast, sympathectomized rats under sleep restriction exhibited decreased stress response (lower serum corticosterone and 11β-HSD1 activity). In addition, they maintained weight loss, explained by a reduced visceral fat pad, leptin, and adiponectin, improved glucose management, and persisting decline in body temperature. These results suggest autonomic nervous system is partially responsible for the WAT-exacerbated stress response and its metabolic and physiological disturbances.

Keywords: Corticosterone, 11β-HSD1, adipose tissue denervation, metabolism, stress

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Introduction

The contemporary lifestyle creates situations that disturb our resting patterns and working and recreational habits. It is estimated that people sleep fewer hours nowadays than in past decades.[1] Epidemiological studies have shown that reduced sleep time/quality is related to developing obesity and other metabolic disorders in infants and adults.[2,3] For instance, a National Health Interview Survey between 2004 and 2007 showed that an increased prevalence of obesity in adults was accompanied by a sleep time decrease (around 30% of consulted persons presented as overweight and slept less than 6 h).[4] Meanwhile, in another meta-analysis study directed at infants and adolescents (between the years

2017 and 2018), 20% of this population sector exhibited an increased overweight but slept less than their recommended 12 h.[5] According to these data, in recent years, the human population tend to suffer an increase in its body mass index at the expense of sleeping less time. In addition, sleep restriction and sleep deprivation (SD) are also associated with high stress levels, increasing plasmatic glucocorticoid concentrations.[6,7] Previous reports have shown that chronic stress (caused by chronic sleep restriction or total SD) triggers an impairment of the hypothalamic–pituitary–adrenal (HPA) axis activity in both humans and rodents, leading to metabolic dysfunctions, such as insulin resistance and augmented central adiposity.[8] Therefore, augmented activity of the HPA-axis has been related directly to an increase of glucocorticoids, which induce a propensity for obesity. In this context, the sympathetic nervous system (SNS) also plays an essential role in sustained stress response adaptations that prone to metabolic disturbance risk. SNS innervating white adipose tissue (WAT) mediates the leptin lipolytic-induced action needed to sustain the energy demand related to stress response.[9] However, exacerbated fat breakdown might contribute to trigger several metabolic issues.[10,11] For example, a stress response might exacerbate lipolysis in the WAT, which increases free fatty acids (FFAs) in a significant manner and is related to worsening glucose handling by inducing insulin resistance in the liver or skeletal muscle.[12] It is in this sense that WAT could be considered a strategic target of study in terms of understanding how stress response derived by sleep less time might be involved in triggering metabolic disturbances.

In addition, glucocorticoids exert a pleiotropic effect over different tissues, which can be activated/inactivated locally by 11β-hydroxysteroid dehydrogenase types 1 and 2 enzymes (11β-HSD1 and 11β-HSD2), respectively.[13] This means that not only circulant glucocorticoid levels are sufficient to explain the impact of the systemic stress response on the distinct potential target tissues. Essentially, 11β-HSD1 enhances glucocorticoids levels by enzymatic reduction of 11-dehydrocorticosterone to corticosterone (cortisol in humans) in organs like the liver, skeletal muscle, and adipose tissue.[13–15] In short terms, 11β-HSD1 activity locally amplifies glucocorticoid levels. For example, 11β-HSD1 action on liver and adipose tissue promotes enhanced gluconeogenesis and lipid storage (or lipolysis, depending on the adipose depot).[14] However, the chronic contribution of 11β-HSD1 on amplified glucocorticoids locally induces several metabolic issues such as obesity, augmented blood pressure, and diabetes mellitus.[16]

11β-HSD1 activity in the adipose tissue is upregulated by cytokines such as tumor necrosis factor (TNF) and interleukin (IL)1 but downregulated by components of the HPA-axis (corticotropin-releasing hormone [CRH] and adrenocorticotrophin hormone [ACTH]).[17] It is also known that the activation of the β-adrenergic receptors by the agonist salbutamol in human mature adipocytes significantly increases 11β-HSD1 activity, strongly suggesting that the SNS might regulate glucocorticoid responses in WAT.[17] Although several reports relate the overexpression of 11β-HSD1 in adipose tissue to hyperglycemia, insulin resistance, obesity, dyslipidemia, and hypertension, 11β-HSD1 knockout animals are resistant to developing obesity and diabetes induced by a high-fat diet.[18,19] As already described, sleep restriction could act as a stressor agent able to alter WAT physiology by a systemic response due to altered HPA-axis. A local stressful response can be installed in visceral WAT by activating the SNS, and in turn, favor the 11β-HSD1 activity.[20] So, we hypothesize that sympathetic visceral WAT denervation could downregulate local and systemic side effects caused by the sleep restriction–mediated stress response. Our results showed that eight weeks of sleep restriction increased serum corticosterone levels and reduced visceral WAT 11β-HSD1 activity, triggering homeostatic alterations such as weight gain and fat-mass loss.

Interestingly, serum leptin levels increased while higher serum adiponectin levels were observed. In addition, the rats presented subtle glucose intolerance and reduced daily body temperature profile. In contrast, the surgical removal of visceral WAT autonomic innervation ameliorated the stress response caused by sleep restriction (lower glucocorticoid levels and 11β-HSD1 activity). Furthermore, it improved the adverse side effects of serum adiponectin and glycemic levels. These results suggest an important WAT-mediated relationship among SNS, glucocorticoid balance, and stress response.

Materials and methods

Animals and general conditions

Male Wistar rats weighing 190–220 g at the beginning of the protocol were provided by the Anatomy Department, Faculty of Medicine, Universidad Nacional Autónoma de México. The animals were individually housed in cages with a 12:12 h light–dark (LD) cycle, lights-on defined as Zeitgeber Time 0 (ZT0), constant temperature (22 ± 1°C), circulating air, and free access to water and food (Rodent Laboratory Chow 5001, IL, USA).

Experimental protocol

The Ethical Committee of the Faculty of Chemical Science at Universidad Autónoma de San Luis Potosí (CEID2014030) approved experiments in strict accordance with the Mexican standards for animal handling (Norma Oficial Mexicana NOM-062-ZOO-1999).

After a week of acclimation, animals were randomly assigned using the R software (version 3.4) to one of four groups: (1) control (SHAM; $n = 8$), *ad libitum* sleep; (2) sleep restriction (SR; $n = 8$), which underwent forced activity for 18 h (16:00 h to 10:00 h following day) and rest for only 6 h per day (10:00 h to 16:00 h); (3) denervation (DX; $n = 8$), which was subjected to WAT denervation and then kept under the same sleep conditions as SHAM; and (4) sleep restriction-WAT denervation (SRDX; $n = 8$), which underwent visceral WAT denervation before being submitted to sleep restriction conditions.

Sympathetic WAT denervation and implant of body temperature sensors

Before the experiments, all animals underwent surgery to insert intra-abdominal temperature sensors (iButton Sensor-Temperature Logger, Maxim Integrated Products, Dallas

Semiconductor, Dallas, TX, USA). In addition, DX and SRDX rats underwent WAT sympathetic denervation. The subjects were anesthetized with 70 mg/kg ketamine (0.1 mL/100 g Anesket, PiSA Agropecuaria, Mexico) and 8 mg/kg xylazine (0.08 mL/100 g Proneticin, Pisa Agropecuaria). A small incision in the abdominal cavity exposed retroperitoneal WAT, which was bilaterally denervated as described by Cantu and Goodman,[21] while epididymal WAT was bilaterally denervated according to Shi *et al*.[22] The same procedure was followed for animals undergoing SHAM surgery, except that the nerve was left intact after visualization. After denervation, the temperature sensors were implanted in the abdominal cavity. The temperature iButtons were programmed to collect temperature data every 60 min during the protocol. Then, the anterior abdominal muscles were sutured by layers with absorbable catgut 000 and abdominal skin with surgical silk for a simple interrupted suture. Intramuscular ketoprofen (3 mg/kg, Vetnco 1 mg/mL) and 3.5 mg/kg gentamicin (Gibco by Life Technologies (Carlsbad, CA, USA); 5 mg/mL) were administered every 24 h for three days to prevent analgesia and infections. After postsurgical recovery week, body weight was monitored to ensure that the animals reached at least the weight gained before surgery. Those that did not were discarded from the experimental protocol.

Sleep restriction protocol

Immediately after recovery, SR and SRDX rats were placed in rotating drums (designed in our laboratory and manufactured by Omnialva SA de CV, Mexico) from ZT9 to ZT3. The drums (33 cm in diameter × 33 cm long) rotated at a speed of 1 rev/3 min, so the rats were forced to stay awake and move without any effort, allowing them to eat and drink *ad libitum*. Each drum was subdivided into four compartments by concentric plates, giving access to four rats simultaneously and individually. Animals were placed in the rotating drum and then returned to their home cages to rest for 6 h from Monday to Friday, simulating human labor itineraries. On weekends, rats remained undisturbed in their home cages. This procedure was carried out for eight weeks.

Food intake and weight gain measure

The remaining food in the home cages was weighed, and new food and fresh water were added when the rats got into the drum for sleep restriction. The body weight gain was calculated weekly when the protocol was restarted (Monday).

Glucose tolerance test and insulin quantification

One week before the end of the experimental protocol (week 7), all animals fasted for 20 h and received intraperitoneal (IP) glucose bolus (1 mg/kg of body weight). In addition, a blood sample was taken (~500 µL) by subtle tail-cut before and 15, 30, 60, 90, and 120 min after glucose bolus administration. Blood samples were centrifuged at 5000 rpm for 10 min, and serum was recovered and stored at −80°C. Glucose concentration was determined using a commercial glucometer (Accu-Chek Performa, Roche, México). At the same time, insulin was measured using a commercial diagnostic enzyme-linked immunosorbent assay (ELISA) kit (Rat insulin ELISA, ALPCO, México).

Tissue and sample collection

At the end of the protocol, each rat received an overdose (65 mg/mL) of pentobarbital (Sedal-Vet, Sedalpharma, Pet's Pharma, México), and iButtons were collected from the abdominal cavity. Blood samples (500 µL) from the inferior vena cava were collected in Eppendorf tubes (1.8 mL) containing a clot-activator gel. The serum was obtained and stored in 50 µL aliquots at −80°C until assay. The epididymal and the retroperitoneal fat pads were dissected bilaterally and weighed. Part of the total adipose tissue was immediately frozen at −80°C, while another portion was placed in 4% paraformaldehyde (PFA). Then, rats were transcardially perfused with 0.9% saline followed by 4% PFA (Sigma-Aldrich Corp., St. Louis, MO, USA) diluted in phosphate buffer (PBS; 0.1 M, pH 7.4).

Metabolic and hormonal assays

Blood samples were collected and processed for triacylglycerides (TG) and cholesterol quantification by colorimetric methods. Hepatic TGs were assessed with a commercial diagnostic kit (TG color GPO/PAP, Wiener Lab, Germany) by quantifying a 4-aminoantipiridine at 500 nm. In contrast, serum cholesterol levels were assessed with a Colestat enzymatic AA kit (Wiener Lab, Germany). Both determinations were calculated using a standard TG and cholesterol concentration reference.

Corticosterone, adiponectin, and leptin serum concentrations were determined with ELISA kits (Corticosterone Elisa Kit Ab108821, Leptin Rat Elisa Kit, and Adiponectin Rat ELISA kit, Abcam, USA).

Tyrosine hydroxylase immunoreactivity

Fixed sections of visceral WAT and brain were removed and embedded in paraffin, and the Ventrolateral Tegmental Area was used as a positive control. Transversal 5 µm sections were obtained in a Microtome (RM2125 RTS, Leica Biosystems, Richmond, IL, USA) and mounted on electro-charged slides (Thomas Scientific, Swedesboro, NJ, USA). For epitope recovery, tissue sections were submerged in Diva Decloaker heat retrieval solution at 100°C in a pressure cooker for 3 min (Biocare Medical LLC, Pacheco, CA, USA). Tissue sections were treated with 0.3% hydrogen peroxide for 15 min and incubated with rabbit antityrosine hydroxylase primary antibody (1:1000; Abcam) overnight in a humidity chamber at room temperature. After three washes with PBS, the goat antirabbit IgG secondary antibody marked with Alexa Fluor 488 was incubated for 2 h (Thermo Scientific, Waltham, MA, USA). Nuclei were visualized using SYTOX orange nucleic acid stain (Molecular Probes, Eugene, OR, USA). Finally, the sections were coverslipped with Vectashield antifade mounting medium (Vector Laboratories, Newark, CA, USA) and visualized in a confocal microscope (LEICA TCS SP2; Leica Microsystems GmbH, Germany). Completeness of the denervation was defined as tissue content of TH less than approximately 30% of the mean value in the sham-operated group.

Table 1. Primer sequences for quantitative PCR.

Gene name	Sequence
LPL (lipoprotein lipase)	Sense: CCAGCTGGGCCTAACTTTGA
NM_012598.2	Antisense: GGAAAGTGCCTCCATTGGGA
Adiponectin	Sense: CAACATTCCGGGGCTCTACT
NM_144744.3	Antisense: TTTGATTCTCGGGGCTACGG
Leptin	Sense: TCACCTGGTTCTTCGGCAAA
NM_013076.3	Antisense: GGGCAAGACCCATCAAGTAGG
11β-HSD1 (11β-hydroxysteroid dehydrogenase type 1)	Sense: GTAGGAGATGCTCAGGAACCCA
NM_017080.2	Antisense: ACTAGCCAACTTCCCTGTCAGA
Pparγ (peroxisome proliferator–activated receptor γ)	Sense: GGGATGTCTCACAATGCCA
NM_013124.3	Antisense: TGGCACCCTTGAAAAATGCG
Gapdh (glyceraldehyde-3-phosphate dehydrogenase)	Sense: GTCATCATCTCCGCCCCTTC
NM_017008.4	Antisense: CATTGAGGAGCAATGCCAGCC

PCR: polymerase chain reaction.

Adipocyte area measurement

Visceral WAT was fixed in paraffin to obtain 5 µm sections mounted in slides as previously described. The slides with tissue were stained following the hematoxylin–eosin technique, and the area was measured on ImageJ for Windows.

Semi-quantitative reverse transcription polymerase chain reaction

According to the manufacturer's recommendations, total RNA was isolated from adipose tissue using Trizol (Invitrogen/Fisher Scientific, Waltham, MA, USA). Equal quantities of total RNA were used to generate complementary DNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Mexico). cDNA samples were also quantified in nanodrop and kept at −20°C until used. The genes *LPL* (lipoprotein lipase), *adiponectin*, *leptin*, *11β-HSD1*, and *Pparγ* (peroxisome proliferator–activated receptor γ) were amplified and analyzed by reverse transcription polymerase chain reaction (RT-PCR). The PCR products were loaded into an agarose gel, and their respective band images were acquired using a Digi Doc-It Imaging system (UVP LLC, Upland, CA, USA). The band's latter quantification was performed using Image J Launcher for Windows (1.4, USA). All data were normalized as the ratio of each product and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) as the housekeeping reference. The respective primer sequences are listed in Table 1.

Activity analysis of 11β-HSD1

To assess 11β-HSD1 activity, the method reported by Luna-Moreno *et al*.[23] was followed, while microsomal WAT was obtained as previously described[24] and transferred into glass tubes containing 700 µL PBS. Blanks were added 100 µL bovine serum albumin solution (1 mg/mL in PBS). Each triplicate set of tubes was preincubated in a water bath at 37°C for 30 min. To initiate the assay, 4 nM NADPH (Sigma-Aldrich Corp.), 100 µL PBS with 3700 Bq (0.1 µCi) [1, 2-^{3}H] cortisone (Perkin Elmer, Waltham, MA, USA), and unlabeled cortisone (Sigma-Aldrich Corp.) was added to each tube to get a final steroid concentration of 100 nM. The tubes were then returned to the water bath for 60 min, and the reaction was terminated by adding 2 mL ice-cold chloroform (J.T. Baker, USA) to each tube. The tubes were centrifuged at 1000*g* for 30 min to separate the organic and aqueous phases. After aspirating the aqueous supernatant fraction, the organic extracts were evaporated overnight at room temperature. Steroid residues were re-suspended in 20 µL ethyl acetate containing either 1 nm corticosterone or cortisone (Sigma-Aldrich Corp.) and resolved by thin-layer chromatography (TLC), using Silica 60 TLC plates (Merck, Germany) in an atmosphere of chloroform 92:8 (v/v) and 95% ethanol (v/v) (Merck KGaA, Germany). The spots corresponding to corticosterone were scraped off, and [^{3}H] corticosterone was quantified in a Bioscan 200 TLC radio chromatogram scanner (LabLogic, Chantilly, VA, USA). Protein concentration was measured following the Bradford[25] method.

Statistical analysis

All data were tested for variance homogeneity with a Kruskal–Wallis test. Two-way analysis of variance (ANOVA) was applied for temperature, glucose test, and insulin curve: one factor was time, and the other was denervation at two levels. One-way ANOVA ($\alpha = 0.05$) was used for the rest of the data. All data were represented as mean value ± standard error of the mean (SEM). A Tukey's post hoc test followed all analyses, and the statistical analysis was performed with GraphPad Prism v 6.0 for Windows (GraphPad Software, USA).

Results

WAT denervation ameliorates local and systemic stress responses related to sleep restriction

At the end of the protocol, all DX ($n = 8$) and SRDX ($n = 8$) animals were tested to confirm that the denervation was correct by tyrosine hydroxylase immunoreactivity (Supplemental Figure 1). A significant difference in corticosterone levels ($F_{3,24} = 81.85$; $P < 0.0001$. Figure 1(A)) was observed after the eight-week protocol. The mean of values of corticosterone in SHAM rats were 150.7 ng/dL. The SR rats showed a significant increase in hormone serum levels (+125% concerning the SHAM; 338 ± 9.9 ng/dL; $P < 0.0001$) despite not having a significant difference in expression ($P = 0.12$), but curiously, it does have a significant difference in activity. In contrast, SRDX presented reduced serum corticosterone

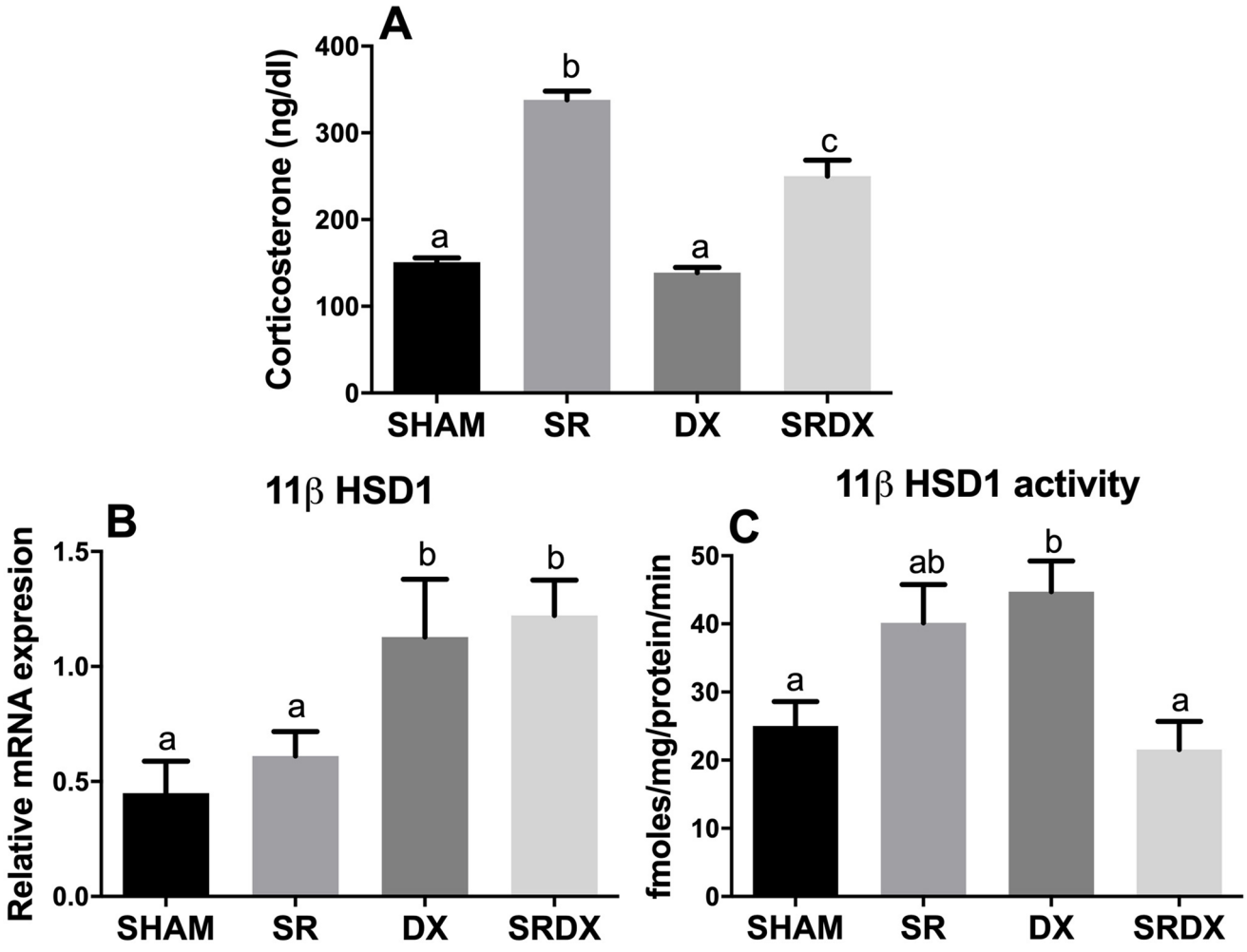

Figure 1. Corticosterone concentration in serum (A) after eight weeks of protocol: control (SHAM; $n=8$), sleep restriction (SR; $n=8$), white adipose tissue (WAT) denervation (DX; $n=8$), and sleep restriction plus WAT denervation (SRDX; $n=8$). Relative mRNA expression of 11β-HSD1 (B) and its protein activity (C) ($n=6$). Results are represented as mean value ± SEM. Different letters show statistical significance ($P<0.05$).

against SR (−25%; 247.7 ± 13.1 ng/dL. $P=0.002$; Figure 1(A)). However, 11β-HSD1 activity in SRDX was equal to that in SHAM ($P=0.12$; Figure 1(C)). Although 11β-HSD1 expression increased ($F_{3,23}=5.94$; $P=0.003$; Figure 1(B)) in both WAT denervated groups (+150%; DX, $P=0.033$; and SRDX, $P=0.0021$; Figure 1(B)), only DX sustained increased 11β-HSD1 activity when compared to SHAM (+180%; $P=0.033$; Figure 1(C)).

Combining WAT denervation and SR reduced body weight gain and adipose fat mass

At the end of the protocol, no differences in food intake ($F_{3,31}=1.37$, $P=0.27$; Figure 2(A)) were observed. Still, body weight gain was reduced in all the experimental groups (around −25%; SR, $P=0.03$; DX, $P=0.03$; and SRDX, $P=0.002$) in comparison to SHAM ($F_{3,23}=5.2$; $P=0.006$; Figure 2(B)).

Visceral WAT weight and the adipocyte area were evaluated to determine whether the mass body reduction was related to modifications in adipose tissue. We observed a significant reduction in visceral fat pads only in SRDX against SHAM ($P=0.03$; Figure 2(C)). In addition, the size of the adipocyte area in visceral WAT pads was analyzed. The results showed that SR and DX presented a smaller cell area than SHAM and SRDX (−55%; $P<0.001$; Figure 2(D)).

Sleep restriction does not affect lipid metabolism

To identify whether adipose alterations are related to disturbances in lipid metabolism, *LPL* and *Pparγ* expression and serum TGs and cholesterol were evaluated. However, no changes were observed in gene expression for both genes ($F_{3,22}=0.54$; $P=0.65$; Figure 3(A) and $F_{3,11}=0.21$; $P=0.88$; Figure 3(B)) nor in serum TG, and cholesterol levels among SR and SRDX rats ($F_{3,44}=1.44$; $P=0.24$; Figure 3(C) and $F_{3,35}=0.92$; $P=0.44$; Figure 3(D)).

Sleep restriction modifies serum levels of leptin and adiponectin

Gene expression and serum levels of leptin and adiponectin were evaluated at the end of the eight-week protocol. The mRNA levels of leptin and adiponectin did not exhibit a significant difference across groups ($F_{3,18}=3.05$; $P=0.054$; Figure 4(A) and $F_{3,22}=2.73$; $P=0.06$; Figure 4(B)). In contrast, the serum leptin concentration in SR ($P=0.002$) and SRDX ($P=0.03$) decreased (around 30%) as compared with SHAM and DX (Figure 4(C)), while only the serum adiponectin concentration increased in SR against the other groups (+35%; $P=0.03$ with SHAM; $P=0.0021$ with DX; and $P=0.0002$ with SRDX; Figure 4(D)).

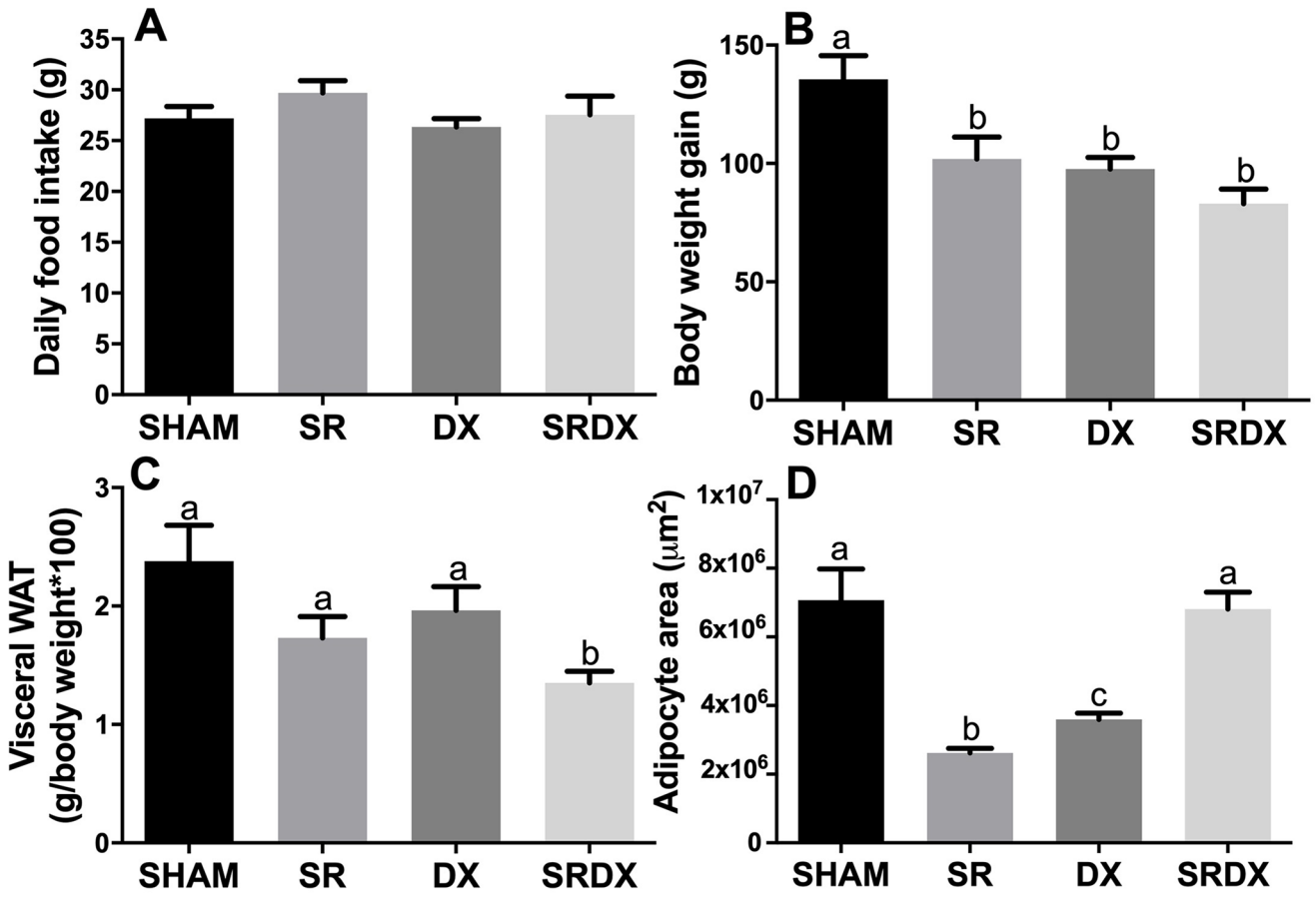

Figure 2. Food intake (A) and body weight gain (B) in SHAM ($n=7$), sleep restriction (SR; $n=8$), denervation (DX; $n=8$), and sleep restriction plus white adipose tissue (WAT) denervation (SRDX; $n=8$). Amount of visceral fat pads (C) and the area of visceral adipocyte (D) at the end of eight-week protocol. Different letters show statistical significance; Graphs show mean value ± SEM ($P<0.05$).

WAT denervation improves glucose tolerance under sleep restriction conditions

After the protocol, a glucose tolerance test (GTT) was applied to identify an alteration in managing of glucose and insulin secretion related to sleep restriction. Both DX and SRDX showed serum glucose comparable to SHAM 15 min after IP injection of glucose bolus, while SR rats exhibited higher glucose levels at the same time point (+30%; $P=0.002$; Figure 5(A)). In addition, DX showed a hyperinsulinemia response against SHAM 15 min (+60%; $P=0.033$) and 120 min (+130%; $P=0.002$) after the glucose bolus was administered (Figure 5(B)).

WAT denervation and the combination with sleep restriction affect body temperature

All groups exhibited a precise circadian rhythm of body temperature (Figure 5(C) and (D)). Interestingly, DX and SRDX showed a significant decrease in the daily temperature profile in the daytime ($F_{3,44}=11.04$; $P<0.0001$) and night ($F_{3,44}=7.84$; $P=0.0003$) periods against SHAM and SR (Figure 5(C) and (D)). In addition, there was a significant decrease in mean temperatures in both DX (−0.4°C; $P<0.0001$ at day; −0.2°C; $P=0.03$ at night) and SRDX ($P<0.0001$; −0.35°C at day and night) (Figure 5(D)).

Discussion

In the present work, we investigated the role of autonomic WAT innervation in the stress-related responses associated with chronic sleep restriction in rats. As expected, eight weeks of sleep restriction triggered a stress response, as shown by a significant increase in serum corticosterone levels.[6,7] Furthermore, compared to the SHAM group, SR presented reduced body weight gain, reduction in visceral fat-pad adipocyte size, and alterations in serum adipokine levels and glucose intolerance. In contrast, WAT sympathetic denervation attenuates the intensity of this stress response in SRDX, partially reducing serum corticosterone and preventing/ameliorating some of the affected parameters, such as adipocyte size, adiponectin levels, and glucose tolerance. These data suggest that stress response promoted by sleep restriction is partly associated with a dysregulation of the autonomic nervous system activity, collaborating with the progression of altered physiological and metabolic processes observed due to chronic sleep restriction.

Sleep restriction and WAT denervation models

To evaluate the stress caused by sleep restriction we used slowly rotating drums (1 rev/3 min), allowing the animals to stay awake, groom, lay down, and avoid extra energy

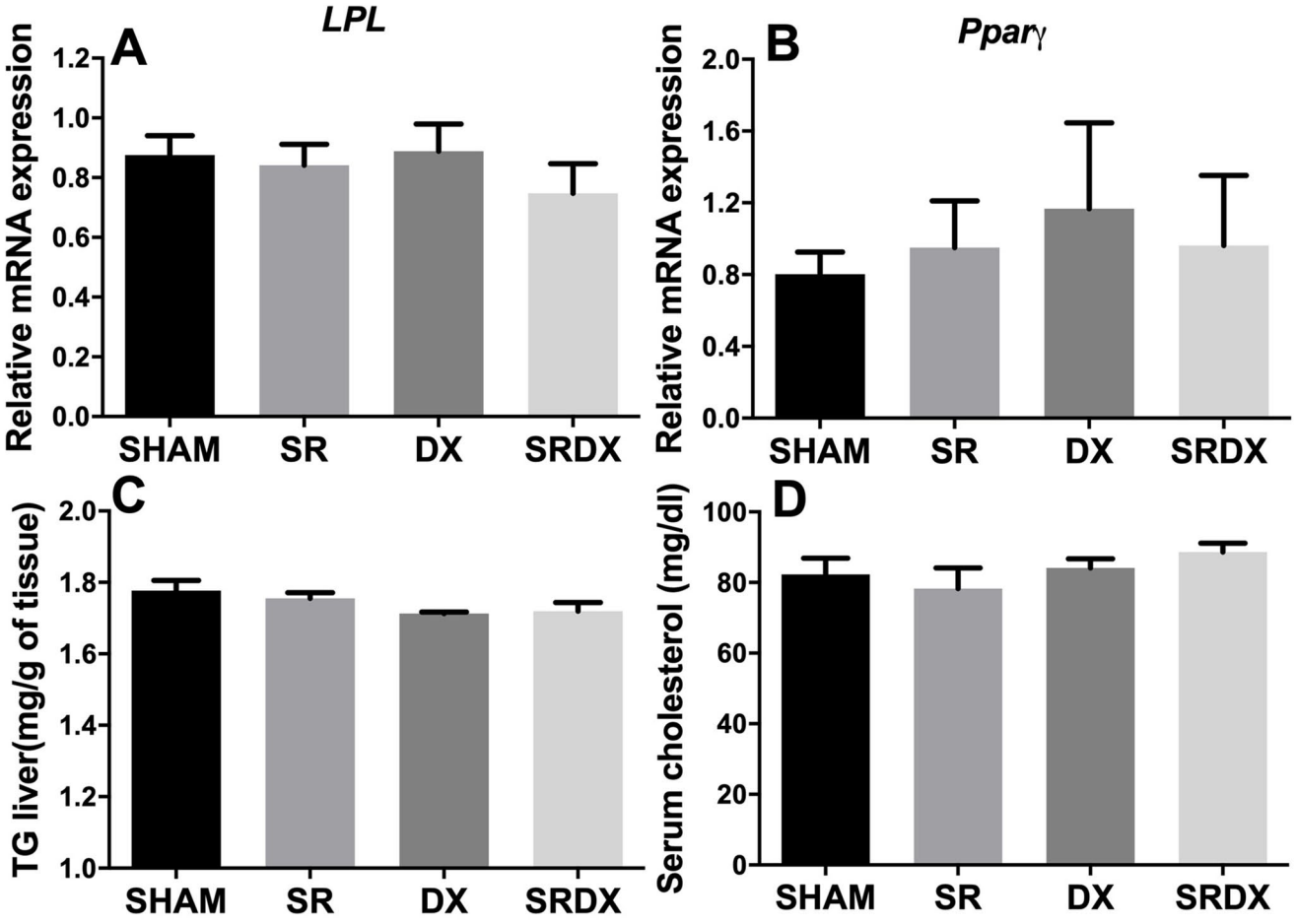

Figure 3. Relative expression of *LPL* (A) and *Pparγ* (B) in visceral adipose tissue at eight weeks of protocol. Triglycerides in the liver (C) and serum cholesterol (D) in controls (SHAM; $n=7$), sleep restriction (SR; $n=7$), denervation (DX; $n=8$), and sleep restriction plus white adipose tissue (WAT) denervation (SRDX; $n=8$). Graphs show mean value ± SEM ($P<0.05$).

expenditure.[26] As a result, the distance traveled per day (18 h) was 37,321 m per animal.

We chose to use this chain of protocols because it is less aggressive, like the popular platform-on-water.[27,28] In addition, animals were free to ingest water and food, and we had more control over measuring how much each animal ate.

WAT denervation ameliorates the stress response elicited by sleep restriction

Several reports show that sleep disturbance is associated with alterations in HPA-axis activity.[7,29,30] These studies have shown that corticosterone plasma levels are the most representative stress response parameter.[31] In agreement with these results, SR rats in our protocol exhibited a significant increase in serum corticosterone (Figure 1(A)). Interestingly, WAT sympathectomy in SR rats reduced circulating corticosterone to control levels. Indeed, SNS plays an essential role in stress response because it represents the nervous component of the HPA-axis and regulatory feedback.[32] Cortisol/corticosterone and sympathetic innervation induce FFAs release from WAT as an energy source.[33–35] Nevertheless, although no changes were observed in serum TG, FFA was expected to be elevated in SR conditions. There is evidence that increased IP infusion of FFA can induce elevated corticosterone in rats, yet the mechanism is poorly understood.[15] We hypothesized that lower FFA in the denervated groups could contribute to reducing corticosterone levels in both. However, further tests, including FFA measurement, are necessary to better understand this event.

To investigate whether sleep restriction induces the local stress response within WAT and the involvement of SNS, we evaluated the expression and activity of 11β-HSD1, which physiologically activates and amplifies glucocorticoid signaling within tissues.[18] Surprisingly, we found that both 11β-HSD1 expression and activity were affected by sleep restriction, suggesting that this protocol is enough to affect the WAT stress response. On the contrary, it is interesting to point out the significant increase in 11β-HSD1 expression and activity in DX, showing that the SNS might play a key role as an inhibitory regulator of this enzyme. Furthermore, it has been reported that 11β-HSD1 expression and activity *in vitro* are affected by β-adrenergic (upregulation) and α2-adrenergic (downregulation) agonist receptors.[9]

Nevertheless, SRDX showed an increase in 11β-HSD1 expression versus SHAM and SR that did not affect the enzymatic activity, suggesting sleep restriction compensates the increase in 11β-HSD1 activity observed in DX. Other

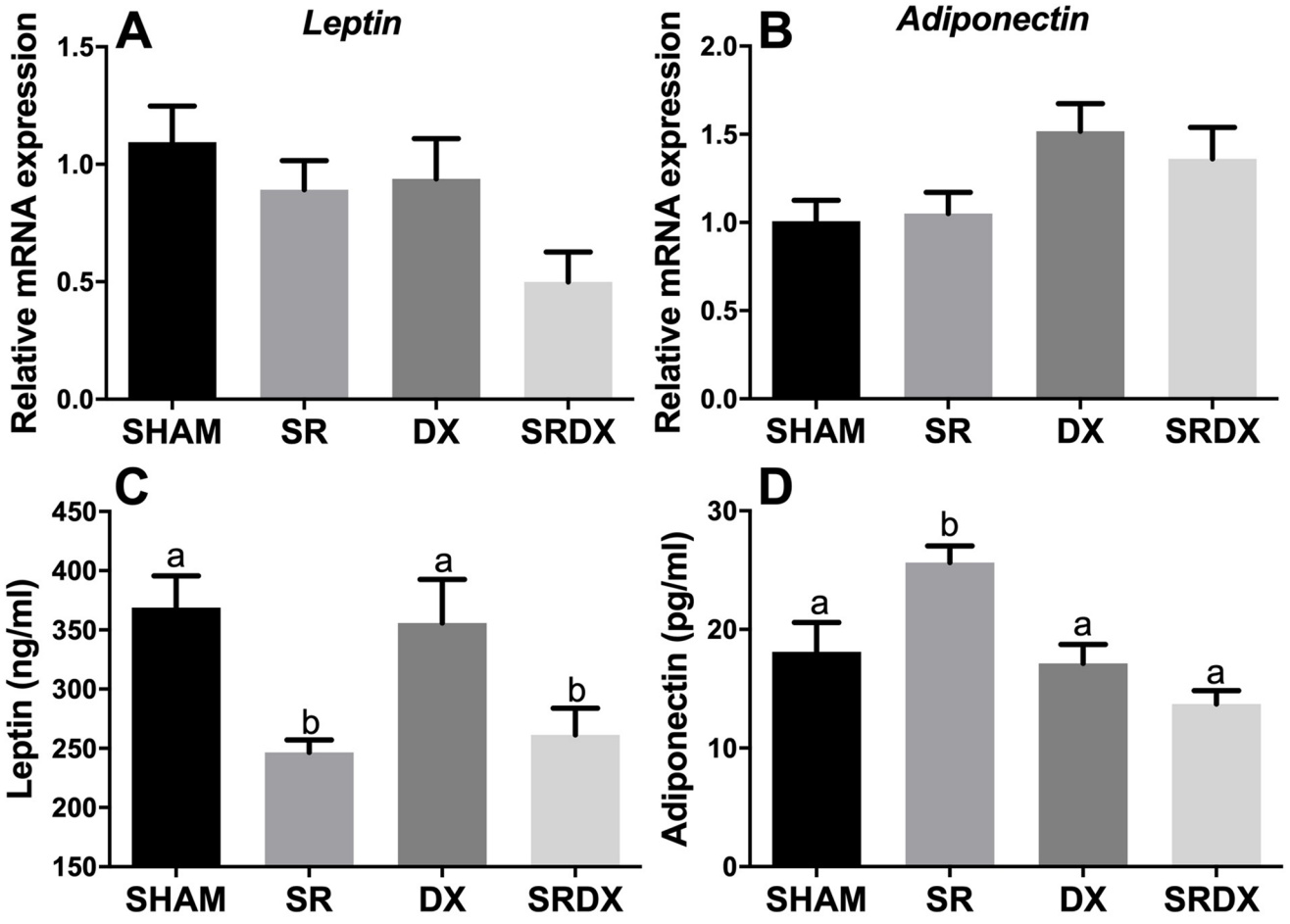

Figure 4. Leptin (A) and adiponectin (B) mRNA levels in visceral WAT. Leptin (C) and adiponectin serum levels (D) in control (SHAM; $n=7$), sleep restriction (SR; $n=8$), denervation (DX; $n=8$), and sleep restriction plus white adipose tissue (WAT) denervation (SRDX; $n=8$). Graphs show mean value ± SEM at eight weeks. Different letters show statistical significance ($P<0.05$).

proinflammatory factors related to sleep disturbances, such as cytokines and hormones, might regulate its activity.[17,36] We hypothesized that the restored 11β-HSD1 activity in SRDX could be indirectly related to corticosterone levels. For example, one of the effects of glucocorticoids is immunosuppression, which could be mediated by cytokines, as TNFα, that can upregulate the glucocorticoid receptor.[36] The reduction in the latter might be related to the decreased enzymatic activity.

Sleep restriction and sympathectomy reduce body weight gain

The literature links chronic sleep disturbances with overweight in humans and rodents.[37,38] Some other reports indicate that altered sleep behavior reduces mass body gain[39–41] and relates an augmented food intake with a high caloric content to sleep restriction.[40,41,42] In this work, we observed that SR rats gained less body mass than SHAM, effect that could be related to a reduced feeding. Barf *et al.*[43] performed a similar SR protocol, except that the diet provided contained 45% fat, despite this, the animal with sleep disturbance also lost weight. Conversely, the rodents in this work were fed a standard diet, ruling out caloric food intake as an additional variable. In addition, dysregulated sleep patterns are associated with deficient nutrient absorption and a decreased growth hormone release, two conditions that might perfectly correlate with impaired anabolism, growth, and mass body gain.[44–46]

We observed that SR and SRDX ate the similar amount of food but displayed a significant decrease in mass body gain compared to controls. These results suggest a negative energy balance likely explained by alterations in anabolic processes or an excessive energy expenditure responsible for weight gain slowdown. Indeed, acute stress exposure is related to several physiological disturbances, including decreased protein and lipid synthesis.[47,48] On the contrary, Barf *et al.*[40] reported that the attenuation in rat body mass gain caused by chronic sleep restriction depends on increased energy expenditure.

The daily body temperature was lower in SRDX and DX than in the control group, discarding the possibility that energy expenditure is responsible. In this sense, stress conditions could affect other tissues; for example, glucocorticoids may hamper muscular and osseous anabolic processes such as proliferation and differentiation.[49] Unexpectedly, the visceral fat accumulation was only significantly reduced in SRDX, potentially related to the weight loss observed in

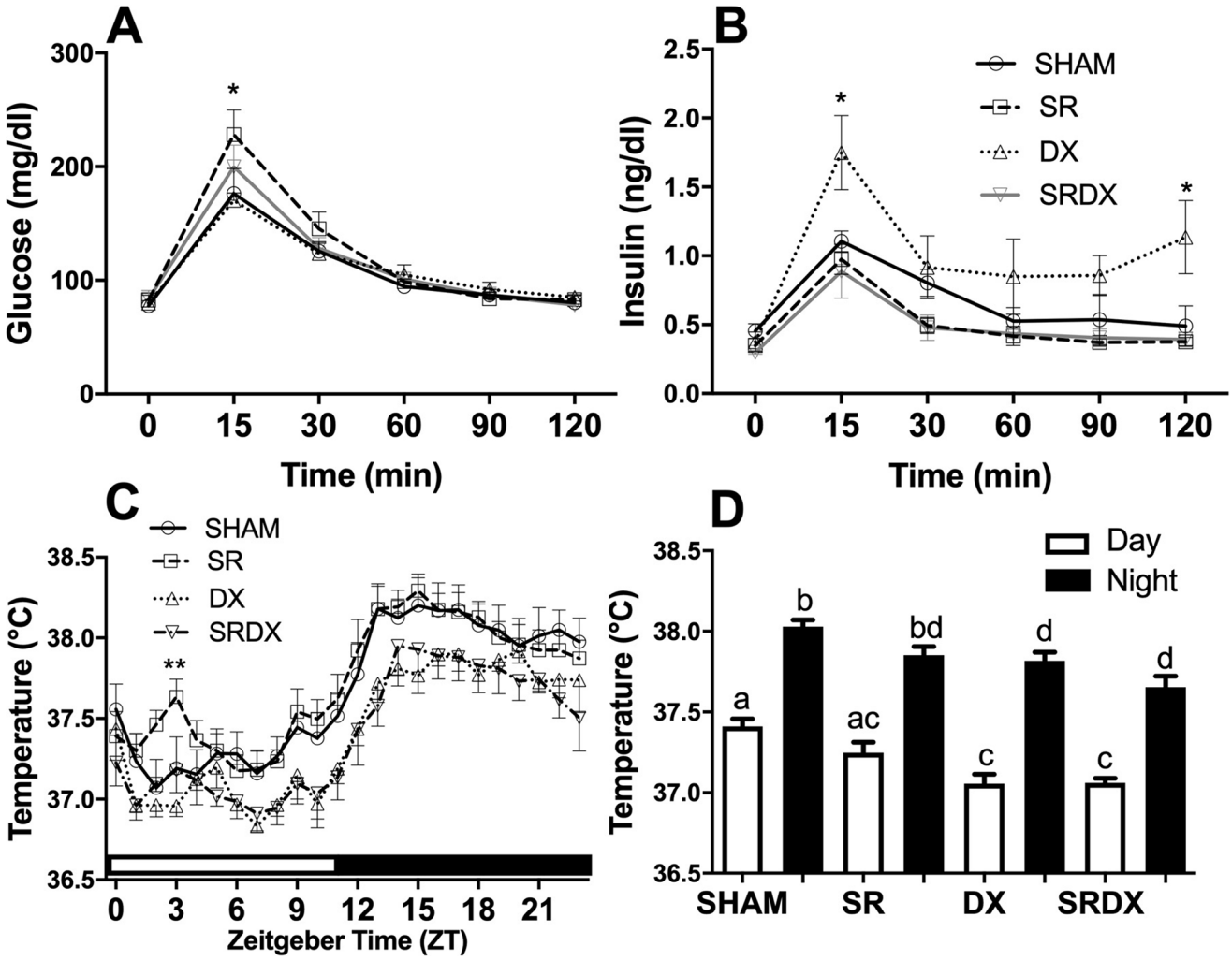

Figure 5. Intraperitoneal glucose test (IPGT). (A) Insulin blood concentrations (B) at the same time as IPGT on controls (SHAM), $n=7$; sleep restriction (SR), $n=7$; denervation (DX), $n=8$; and sleep restriction plus white adipose tissue (WAT) denervation (SRDX), $n=8$. Daily temperature curves (C) for controls (open circles), sleep restriction (open square), denervation (open triangle), and sleep restriction plus WAT denervation (open down triangle), $n=8$; $^{*}P<0.05$ represents the difference against the control group. The empty transversal bar represents the light phase, and the black transversal bar represents the night phase; $n=8$ for all groups (D). Graphs show mean value ± SEM at eight weeks. Different letters show statistical significance with a $P<0.05$.

these animals. Our results showed that mass in denervated pads was reduced under sleep restriction conditions against SHAM, but the opposite was observed in SRDX versus SHAM. This effect contradicts previous works in which fat pad increment or proliferation under bilateral or unilateral surgical methods is reported without stress.[50–52]

The stereological analysis of visceral WAT cells showed that adipocyte areas in SR and DX were significantly smaller than in the other groups, indicating a decrease in adipocyte lipid contents. However, there was no significant reduction in SRDX adipocyte area, suggesting that both denervation and sleep restriction conditions could act as a kind of emergent property.

Sympathectomy does not affect WAT genetic expression and lipid values

Both SR and WAT denervation caused a decrease in body weight gain and adiposity in SRDX and lipid content in SR and DX adipocytes. Thus, the expression of critical elements involved in lipid metabolism was evaluated. It is known that LPL is an enzyme essential to lipid absorption from the circulation, while the PPARγ is involved in adipogenic and inflammatory processes, adipocyte differentiation, and proliferatio.[53,54] Unexpectedly, there were no changes in the expression of both genes; still, it is essential to consider that PPARγ expression obeys multiple conditions, including postprandial and circadian regulation.[55,56] In our model, food access was not restricted, and tissue sampling was always performed at the same time (around midday), so circadian expression changes were not likely. Since the adipocyte area showed a significant decrease due to either SR or adipose denervation, we argue that possible alterations in LPL and PPARγ expression might occur at a different time than the one currently analyzed.[55,57] In addition, we investigated whether the reduction in lipid content observed in the adipocyte area in SR and DX resulted in dyslipidemia or hepatic lipid accumulation. Therefore, we measured serum lipids (TG and cholesterol) and hepatic TG content, but no evidence of alterations in these metabolites was found, hence

the possibility of developing dyslipidemia or hepatic steatosis was discarded.

Sleep restriction alters adipokine secretion

Since the discovery of leptin as a cytokine secreted by WAT, numerous adipokines have been identified. This tissue went from being a mere lipid store to constituting an endocrine organ.[58] Some studies argue that fat mass alteration modifies the amount of adipokines secreted.[59] Leptin expression and secretion occur postprandially in response to insulin-promoted satiety and thermogenesis.[60] Adiponectin promotes insulin sensitivity, decreases hepatic glucose production, and favors lipid oxidation.[61] In agreement with previous reports, our results showed a significant decrease in serum leptin in SR and SRDX groups compared to SHAM group.[62] The reduced leptin in SR might depend on increased corticosterone, which modifies adipocyte transcriptional patterns and metabolism.[63] In turn, leptin inhibits CRH and corticosterone release, suggesting an imbalance in the adipokine-glucocorticoids regulation loop.[64] On the contrary, the release of adiponectin in SR group increased significantly. In humans, this effect has been observed in response to prednisone administration; it maybe can be a mechanism to compensation of glucocorticoids levels due to sleep restriction like stressor factor.[65]

An alternative but not explored aspect in the present research is related to adipose tissue inflammatory response as part of the consequences of chronic stress conditions. As previously reported, sleep disturbance (a decrease in sleep duration or SD) is associated with increased proinflammatory markers (at systemic and adipose tissue levels) such as TNFα, IL6, IL1α, and IL1β.[66,67] In this context, it is known that elevated leptin levels increase the production of TNFα and IL6 cytokines, while adiponectin acts as an anti-inflammatory factor.[67,68] However, according to our results, the SR group exhibited decreased leptin and increased adiponectin levels, suggesting that this immune response might be ameliorated under this stress response condition. Hence, it will be interesting to continue this study by exploring the levels of these inflammatory markers in both SR and SRDX groups, respectively.

Sympathetic denervation of the WAT can improve glucose tolerance in the sleep restriction conditions

Many reports have shown that SR increases glycemia due to chronic glucocorticoid secretion, which impairs the insulin action.[69] For instance, SR rats showed a significant increase in glycemia 15 min after GTT. However, WAT denervation improved glucose management since SRDX did not display this same glycemic pattern. This result could be associated with the corticosterone levels measured in SRDX animals, which promote insulin sensitivity and improve glucose management. On the contrary, DX showed a significant increase in insulin levels, mainly 15 and 120 min after IP glucose administration. This result could be related to the 11β-HSD1 activity observed in DX since a positive relationship between adipose enzymatic activity and insulin alterations has been described. However, this phenomenon is yet to be completely understood.[70] The data suggest that, under SR, sympathetic denervation can improve glucose tolerance without altering insulin sensibility, likely associated with restored 11β-HSD1 activity, and thus the insulin pathway remains unaltered.

WAT denervation decreases daily body temperature profile

Body temperature is one of the several parameters modulated by the autonomic nervous system. Temperature control is in the hypothalamus, which regulates the mechanism leading to thermogenic balance and the sympathetic and parasympathetic branches to lose or generate heat as required.[71] Body temperature increases due to metabolic activity, like FFA oxidation by brown adipose tissue (BAT) and FFA release by WAT.[72] Our results show that DX and SRDX animals had a lower body temperature than those not denervated. This effect agrees with the reports describing that WAT sympathetic innervation participates in core temperature upregulation.[73]

Furthermore, a lower body temperature has been reported in human and rodent models under sleep alterations even after only one night. However, in our study, the body temperature of SR rats was not reduced after the eight-week protocol. Still, we do not rule out the possibility that temperature alterations could be observed if SR time is increased.[74]

While this study was successful, some things could be improved. First, it would be helpful to analyze the effect of sleep restriction at other times of the day and during extended periods of deprivation. In addition, analyzing some markers of inflammation in adipose tissue or serum could provide a more comprehensive perspective on inflammation associated with sleep restriction and increase the sample size to enhance the study's statistical power. Despite this, the results could be the starting point for planning more detailed experiments to enlighten which intercellular and intracellular signaling systems are altered in the metabolic conditions associated with sleep restriction.

In conclusion, the present work suggests that sleep restriction causes systemic stress response and can alter adipose tissue metabolism, adipokine release, and some systemic metabolic variables. Sympathetic innervation in visceral WAT impacts systemic glucocorticoid release, homeostatic response to glucose, and adipocyte physiology. Our results help to understand some aspects of pathologic effects generated by poor sleep quality and stress, variables essential to a modern lifestyle where a saturated work schedule and night recreation are common occurrences.

AUTHORS' CONTRIBUTIONS

All authors approval the final version of manuscript. LEA-A, ABR, and RS-D contributed to study design. LEA-A, ODRP, and SC-R contributed to animal handling. LEA-A and ABR contributed to data analysis and collection. LEA-A, NS, ABR, MD-M, and RS-D contributed to data interpretation. LEA-A, NS, ABR, MD-M, and RS-D contributed to manuscript drafting. LEA-A, NS, ABR, ODR-P, SCR, MD-M, OF-S, and RS-D contributed to manuscript edition and revision. MD-M contributed with reagents/analytical tools.

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SUPPLEMENTAL MATERIAL

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