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Chronic stress combined with fructose diet reduces hypothalamic insulin signaling and antioxidative defense in female rats

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Short Title**:** Stress and fructose affect hypothalamic insulin signaling in female rats

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Key words**:** Glucocorticoid receptor, leptin, appetite, female rats, inflammation, insulin.

Abstract

***Background*:** Increased fructose consumption and chronic exposure to stress have been associated with development of obesity and insulin resistance. In the hypothalamus, a crossroad of stress response and energy balance, insulin and glucocorticoids regulate expression of orexigenic neuropeptides – neuropeptide Y (NPY) and agouti-related protein (AgRP) and anorexigenic neuropeptides – proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). ***Objectives*:** We investigated whether chronic stress and fructose diet disrupt these hormones' signaling pathways and appetite control in the hypothalamus, contributing to development of insulin resistance and obesity. Potential role of hypothalamic inflammation and oxidative stress in development of insulin resistance was also analyzed. ***Methods*:** Insulin, glucocorticoid and leptin signaling, expression of orexigenic and anorexigenic neuropeptides, and antioxidative and inflammatory status in the whole hypothalamus of fructose-fed female rats exposed to unpredictable stress for 9 weeks were analyzed using qPCR and Western blot. ***Results*:** Chronic stress combined with fructose-enriched diet reduced protein content and stimulatory phosphorylation of Akt kinase, and elevated 11β-hydroxysteroid dehydrogenase 1 and glucocorticoid receptor expression, while alterations in the appetite regulation (NPY, AgRP, POMC, CART, leptin receptor, and SOCS3 expression) were not observed. The expression of antioxidative defense enzymes (mitochondrial manganese superoxide dismutase 2, glutathione reductase and catalase) and proinflammatory cytokines (IL-1β, IL-6 and TNFα) was reduced. ***Conclusions*:** Our results underline the combination of long-term stress exposure and fructose overconsumption as more detrimental for hypothalamic function than either of the factors separately, as it enhanced glucocorticoid and impaired insulin signaling, antioxidative defense and inflammatory response of this homeostasis-regulating center.

Introduction

Increased fructose consumption and pervasive exposure to stress represent inevitable burden of modern lifestyle. Fructose overconsumption has been associated with development of obesity and insulin resistance in both humans [1] and animals [2]. Likewise, glucose intolerance was observed in rats exposed to chronic stress [3]. In support, the majority of diabetic patients experienced significantly higher exposure to chronic stress compared to subjects with normal glucose tolerance [4]. This raises the need for understanding molecular mechanisms by which fructose and stress disturb metabolism.

Hypothalamus is the key regulator of stress response and energy balance including food intake and energy expenditure. Two important signaling pathways contributing to these processes are mediated by insulin – one of the satiety signals, and glucocorticoid hormones – playing multiple roles in this brain region including regulation of stress response, energy balance and inflammation. In addition, glucocorticoids have the ability to downregulate insulin signaling in the brain [5]. It is of note that obesity is not associated with hypercortisolemia, but rather with elevated tissue-specific intracellular regeneration of active glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 (HSD1) [6] and hexose-6-phosphate dehydrogenase (H6PDH), which provides a cofactor for the reaction.

A significant association between hypothalamic insulin resistance on one hand and obesity and diabetes on the other has been postulated both in humans [7] and rodents [3, 8]. Fructose overconsumption [2] as well as chronic exposure to stress [3] have been described to reduce total protein kinase B (Akt), Ser^{473}-phosphorilated Akt (pAkt-Ser^{473}), and insulin-stimulated tyrosine phosphorylation of insulin receptor in the hypothalamus.

Within the hypothalamus, insulin and glucocorticoids regulate expression of neuropeptide Y (NPY) and agouti-related protein (AgRP), orexigenic neuropeptides that stimulate food intake and reduce energy expenditure. Insulin inhibits expression of these neuropeptides. Thus, reduced insulin signaling disables proper regulation of energy homeostasis leading to AgRP/NPY overexpression and consequently, to increased appetite and body weight [9]. On the other hand, glucocorticoids can directly stimulate expression of NPY and AgRP and functional glucocorticoid receptor (GR) binding sites were found in their promotors [10]. Two anorexigenic neuropeptides, proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) are also expressed in the hypothalamus and positively regulated by insulin [11] and glucocorticoids [12].

AgRP/NPY and POMC neurons are also regulated by leptin, another satiety signal [13], which is secreted by adipose tissue in favorable energy conditions, but acts through leptin receptor (ObRb) in the hypothalamus. Leptin plasma concentration is directly proportional to the mass of adipose tissue – it is

increased in obesity while it decreases with the weight loss [13]. Nevertheless, hyperleptinemia can cause leptin resistance, mainly through downregulation of ObRb and/or the induction of a feedback inhibitor – suppressor of cytokine signaling 3 (SOCS3) [13], resulting in increased appetite.

Fructose consumption was shown to down-regulate POMC mRNA in the hypothalamus [14], but literature data regarding fructose effects on NPY and/or AgRP expression are controversial, ranging from stimulatory to inhibitory [15]. Similarly, daily exposure to psychosocial stress was associated with the development of metabolic syndrome [16], but the correlation between stress and obesity remains unclear. Studies show that, when exposed to chronic stress, some people avoid food, while others reach for highly palatable, "comfort", food abundant with carbohydrates and fats [9]. Prevalence to comfort food is also observed in animals exposed to this type of food during stress exposure [17].

Oxidative stress is considered as one of the mechanisms contributing to development of obesity and insulin resistance [18]. The brain is vulnerable to oxidative damage because of low content of antioxidants [19] and oxidative stress in the hypothalamus has been strongly implicated in development of insulin and leptin resistance [20]. Both high-fructose diet [21] and chronic stress [22] were shown to disturb expression of antioxidative enzymes including cytoplasmic copper-zinc superoxide dismutase 1 (SOD1), mitochondrial manganese superoxide dismutase 2 (SOD2), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red) and catalase (CAT).

Inflammation is also implicated in metabolic disorders [23], since it can disrupt hypothalamic insulin signaling altering the regulation of energy homeostasis. It is postulated that proinflammatory cytokines IL-1β [24] and TNFα [25] can induce inhibitory phosphorylation of insulin receptor substrate 1 (IRS1) on Ser^{307} residue (pIRS1-Ser^{307}), inhibiting kinase activity of insulin receptor. High fructose consumption has a potential to activate nuclear factor-κB (NFκB) signaling pathway in the hypothalamus elevating proinflammatory cytokines [2, 26]. On the other hand, data on stress-related perturbations in hypothalamic inflammatory status are rather conflicting. Expression of proinflammatory cytokines IL-1β, IL-6, and TNFα was reported to be both elevated [27] and unchanged [28] in the hypothalamus of male rats after chronic exposure to different kinds of stressors.

Most of the studies investigating either the effects of fructose or the effects of stress on hypothalamic regulation of metabolic homeostasis are performed in males. However, there are about two million more obese women than men in the United States, and similar data were reported for developing countries in the South-East of Asia [29, 30], emphasizing the importance of studies on females.

Hypothalamus is a crossroad of stress response and energy balance regulation. Data on mechanisms by which stress and fructose exert deleterious metabolic effects are somewhat ambiguous and although these environmental factors commonly accompany each other, only a few studies address the

consequences of their combination. With this in mind, we tested the hypothesis that long-term exposure to stress in combination with fructose-enriched diet disrupts insulin and glucocorticoid signaling, and related appetite control in the hypothalamus of female rats, contributing to development of insulin resistance and obesity. In addition, we investigated oxidative stress and inflammation as potential mechanisms contributing to insulin resistance in the hypothalamus. To achieve these goals, we analyzed insulin, glucocorticoid and leptin signaling, as well as the antioxidative and inflammatory status in the hypothalamus of fructose-fed female rats exposed to chronic unpredictable stress.

Materials and Methods

Material

Fructose was purchased from Apipek (Bečej, Serbia). Anti-HSD1 (ab109554), anti-SOD1 (ab13498), anti-SOD2 (ab13533), anti-GSH-Red (ab16801), anti-CAT (ab16731) and anti-GSH-Px (ab22604) primary antibodies, secondary anti-mouse and anti-rabbit IgG H&L horseradish peroxidase (HRP)-linked antibody (ab97046) and (ab6721), respectively, were obtained from Abcam (Cambridge, UK), anti-GR (H-300; sc-8992), anti-H6PDH (sc-67394), anti-NFκB/p65 (C-20; sc-372), anti-IκB (sc-371), anti-ObRb (sc-8391), anti-SOCS3 (H-103; sc-9023), anti-pAkt-Ser^{473} (sc-7985-R), anti-pAkt-Thr^{308} (sc-16646-R), anti-Akt (sc-8312), anti-IRS1 (E-12; sc-8038) and anti-pIRS-1-Ser^{307} (sc-33956) from Santa Cruz Biotechnology, and anti-β actin antibody (AC-15) and GAPDH (G9545) from Sigma Chemicals (St. Louis, MO, USA). Immobilon-FL polyvinylidenedifluoride (PVDF) membrane was a product of Millipore, USA, while Amersham ECL Western Blotting Detection Kit was acquired from GE Healthcare Life Sciences. Leptin High Sensitivity EIA kit was obtained from (Millipore, USA). High capacity cDNA reverse transcription kit, RNase inhibitor, TaqMan® Universal PCR Master Mix with AmpErase UNG, and TaqMan® Gene Expression Assay primer-probe mix for: leptin (Rn00565158_m1), SOCS3 (Rn00585674_s1), AgRP (Rn01431703_g1), NPY (Rn01410145_m1), ObRb (Rn00561369_m1), IL-1β (Rn00580432_m1), IL-6 (Rn01410330_m1), TNFα (Rn01525859_g1), and hypoxanthine phosphoribosyl transferase 1 (HPRT1) (Rn01527840_m1) were all products of Applied Biosystems. Power SYBR® Green PCR Master Mix was purchased from Applied Biosystems, and specific primer pairs for: POMC: F 5'-TCC ATA GAC GTG TGG AGC TG-3', R 5'-GAC GTA CTT CCG GGG ATT TT-3'; CART: F 5'-GCC CTG GAC ATC TAC TCT GC-3', R 5'-CAC TGC GCA CTG CTC TCC-3' and HPRT: F 5'-CAG TCC CAG CGT CGT GAT TA-3', R 5'-AGC AAG TCT TTC AGT CCT GTC-3' from Invitrogen. TRIzol®Reagent (AmBion), RNase free DNase I (Ferments), and RNase-DNase free water (Eppendorf) were also used.

Animals and Treatment

Female Wistar rats (2.5 months old), bred in our laboratory, were randomly divided into four experimental groups during the 9-week treatment: a control group fed with commercial standard chow and drinking water, a fructose group fed with the same chow and 10% (w/v) fructose solution instead of drinking water, a stress group that was fed like the control group and exposed to unpredictable sequence of stressors, 1 or 2 per day for 9 weeks, and a stress + fructose group, which was fed like the fructose group and also exposed to stress. The stress protocol was a modified protocol of Joels and colleagues [31] and included following stressors: forced swimming in cold water for 10 min, physical restraint for 60 min, exposure to a cold room (4°C) for 50 min, wet bedding for 4 h, switching cages for 2 h, rocking cages for 1 h, and cage tilt (45°) overnight. The number (1 or 2) and type of daily stressor(s), as well as the onset of stress exposure (between 4 pm and 7 pm for the overnight cage tilt, and between 9 am and 4 pm for all the other stressors) were randomly selected at the beginning of the treatment. A particular stressor was never applied in two consecutive days or twice in a day. All experimental groups had ad libitum access to food and drinking fluid during the treatment period. Animals (12 per group) were housed three per cage and kept under standard conditions, at 22°C with a 12-h light/dark cycle. Chow [g/(rat·d)] and fluid [ml/(rat·d)] intake was determined daily by measuring the intake per cage and dividing by the number of rats housed in the cage (three). This was then used to calculate daily energy intake as follows: energy intake for control rats was calculated as calories ingested as chow [chow weight (g)×11 kJ], while energy intake for fructose-fed rats was calculated as sum of calories ingested as chow and fructose solution [chow weight (g)×11 kJ + fructose intake (ml)×1.72 kJ]. All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade. Permit number: 02-11/14.

Plasma Parameters

Animals were sacrificed by rapid decapitation after overnight fasting during which experimental animals were provided only with drinking water. All animals were sacrificed in diestrus phase of estrous cycle, which was determined from analyses of vaginal smears. For plasma preparation, the trunk blood from each experimental animal was collected in the separate EDTA-coated tube and centrifuged at 3,000 rpm for 10 min. Plasma was stored at -20°C until use. Leptin plasma concentrations were measured by Rat Leptin ELISA kit according to manufacturer's instructions.

The Preparation of hypothalamic tissue extract

After decapitation, the hypothalamus was excised from the ventral side of the brain having the thalamus as the dorsal limit, the optic chiasm as the rostral, and the mammillary bodies as caudal limit. Excised hypothalami were snap frozen and kept in liquid nitrogen until use. After thawing, hypothalami were homogenized in ice-cold RIPA buffer 1:4 (w/V) (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 10 mM EDTA-Na_2, 10 mM EGTA-Na_2, 0.5% Triton X, 1% NP40, 0.1% SDS, 2 mM dithiothreitol, and protease and phosphatase inhibitors) with 20 strokes of glass homogenizer. Homogenates were sonicated 3×5 s, 1A, 50/60 Hz on ice, incubated on ice for 30 min with frequent vortexing, and centrifuged 20 min on 14000xg, 4ºC. The obtained supernatants were used as the hypothalamic tissue extracts.

SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Samples were mixed 1:1 with 2x Laemmli's buffer and boiled for 5 min. Proteins (50 µg) were separated by electrophoresis through SDS polyacrylamide gels and transferred onto PVDF membrane. To detect proteins involved in glucocorticoid, leptin and insulin signaling, as well as in inflammation and antioxidative defense, membranes were incubated with appropriate primary antibodies, followed by HRP-conjugated secondary antibodies (1:30,000). For correction of protein load, membranes were probed with anti-β actin primary antibody followed by respective HRP-conjugated secondary antibody. Immunopositive bands were visualized by the ECL reaction. Quantitative analysis of immunoreactive bands was performed using ImageJ software.

RNA Extraction and Reverse Transcription

Total RNA was extracted from hypothalami (50-100 mg) after thawing using TRIzol®Reagent following the manufacturer's protocol. RNA was dissolved in 30 µl of RNase-DNase free water and RNase inhibitor was added. Concentration and purity were tested spectrophotometrically (OD 260/280 > 1.8 was considered satisfactory). RNA integrity was confirmed by 1% agarose gel electrophoresis. Prior to cDNA synthesis, DNA contamination was removed by DNAse I treatment (Fermentas), according to the manufacturer's instructions. cDNA was synthesized from 2 µg of RNA. The reverse transcription was performed in a 20 µl reactions with MultiScribe™ Reverse Transcriptase in the presence of Random Primers using High Capacity cDNA Reverse Transcription kit. Reactions were carried out under RNase free conditions at 25°C for 10 min followed by 37°C for 2 hours and final denaturation at 85°C for 5 min. The cDNA was stored at -80°C until further use.

Real Time PCR

The expression of orexigenic neuropeptides and proinflammatory cytokines was analyzed by TaqMan qPCR and the expression of anorexigenic neuropeptides was analyzed by SYBR® Green qPCR using AB Prism 7,000 Sequence Detection System. All reactions were performed in 25 µl volume in triplicates and mean Ct value for each triplicate was used for further analysis. TaqMan reaction mix consisted of 1 × TaqMan® Universal PCR Master Mix, with AmpErase UNG, 1 × TaqMan® Gene Expression Assay and cDNA template (20 ng of RNA converted to cDNA). SYBR® Green reaction mix consisted of 1x Power SYBR® Green PCR Master Mix, specific primer sets and cDNA template. Thermal cycling conditions were: (2 min incubation at 50°C for UNG activation), 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The specificity of SYBR® Green reaction was verified by melt curve analyses. No template control was included for each target gene to detect possible reagent contamination. Relative quantification of gene expression was performed using comparative $2^{-\Delta\Delta Ct}$ method. HPRT1 was used as reference gene.

Statistical Analysis

To determine the effects of fructose and stress treatment, as well as their interaction, two-way ANOVA followed by the post-hoc Tukey test was used. A probability level less than 0.05 was considered to be statistically significant.

Results

Energy Intake

Energy consumed daily did not differ between experimental groups (Table 1).

Hypothalamic Insulin and Glucocorticoid Signaling

The influence of fructose-enriched diet and stress on insulin signaling in the hypothalamus was examined at the level of IRS1 and Akt. Total IRS1 and the level of pIRS-Ser^{307} as well as their ratio were not altered by stress, fructose or their combination (Fig. 1). However, for the total Akt protein abundance and the level of pAkt-Ser^{473} the main effect of fructose (respectively: [$F(1,12) = 28.44$, $P < 0.001$] and [$F(1,12) = 43.19$, $P < 0.0001$]), stress ([$F(1,12) = 22.12$, $P < 0.001$] and [$F(1,12) = 32.00$, $P < 0.0001$]) and their interaction ([$F(1,12) = 5.60$, $P < 0.05$] and [$F(1,12) = 25.96$, $P < 0.001$]) were detected. Additionally, the main effects of stress [$F(1,12) = 12.49$, $P < 0.01$] and fructose [$F(1,12) = 30.21$, $P < 0.001$] were found on the level of pAkt-Thr^{308}. All three parameters were significantly reduced in fructose-fed stressed animals compared to other experimental groups, *i.e.*: to control ($P < 0.001$), to fructose-fed ($P < 0.05$ for

pAkt-Thr^{308}, $P < 0.001$ for pAkt-Ser^{473} and $P < 0.01$ for total Akt) and to stressed rats on standard diet ($P < 0.01$ for pAkt-Thr^{308} and $P < 0.001$ for pAkt-Ser^{473} and total Akt). The ratio of each phosphorylated form to total Akt remained unaltered.

Prereceptor metabolism of glucocorticoid hormones was analyzed at the level of HSD1 and H6PDH protein (Fig. 2). Two-way ANOVA showed the main effect of fructose [$F(1,11) = 9.44$; $P < 0.05$], stress [$F(1,11) = 7.64$; $P < 0.05$], and their interaction [$F(1,11) = 7.19$; $P < 0.05$] on HSD1. The protein level of HSD1 was elevated in fructose-fed rats exposed to stress in respect to untreated and fructose-fed unstressed animals ($P < 0.01$), and to stressed animals on standard diet ($P < 0.05$). Similarly, H6PDH was elevated in animals exposed to both stress and fructose diet in comparison to all other experimental groups (control: ($P < 0.05$), fructose: ($P < 0.05$) and stress ($P < 0.01$)) as a consequence of fructose effect [$F(1,12) = 10.65$; $P < 0.01$] and its interaction with stress [$F(1,12) = 12.74$; $P < 0.01$].

When GR protein level was analyzed using Western blot method (Fig. 2) a significant effect of stress [$F(1,12) = 7.40$; $P < 0.05$], fructose [$F(1,12) = 6.88$; $P < 0.05$] and their interaction [$F(1,12) = 7.63$; $P < 0.05$] was observed. Post-hoc test determined increase in GR protein level in hypothalamus of fructose-fed rats exposed to stress in comparison to all other experimental groups ($P < 0.05$).

Leptin Signaling and the Expression of Orexigenic and Anorexigenic Neuropeptides

Examination of leptin signaling included quantification of leptin plasma concentration, and ObRb and SOCS3 expression level. A significant effect of stress was detected on plasma leptin concentration [$F(1,33) = 25.8$; $P < 0.0001$] as it was decreased in both stressed groups ($P < 0.01$ for stressed on standard diet in respect to the control group, and $P < 0.05$ for stressed on fructose diet in respect to the control and fructose-fed unstressed animals) (Table 1). While protein and mRNA levels of ObRb were not affected by any of the applied treatments (Fig. 3A), a significant effect of stress [$F(1,20) = 223.42$; $P < 0.0001$] and fructose [$F(1,20) = 15.51$; $P < 0.001$] on SOCS3 expression was reflected in decreased mRNA level in both stressed groups in comparison to the control one ($P < 0.001$). Additionally, SOCS3 mRNA level was lower in stressed fructose-fed rats compared to rats exposed solely to fructose diet ($P < 0.001$) and stress ($P < 0.05$). Similarly, SOCS3 protein level was decreased in stressed animals on fructose diet compared to the control and fructose-fed unstressed animals ($P < 0.05$) and to stressed animals on standard diet ($P < 0.01$), resulting from a significant effect of stress [$F(1,12) = 5.34$; $P < 0.05$], fructose [$F(1,12) = 10.14$; $P < 0.01$], and their interaction [$F(1,12) = 5.87$; $P < 0.05$] (Fig. 3B).

Fructose and stress, applied alone or in combination, had no statistically significant effect on the mRNA level of orexigenic (NPY and AgRP) or anorexigenic (POMC and CART) neuropeptides (Fig. 3C).

Hypothalamic Antioxidative Defense and Inflammatory Status

When protein level of antioxidative enzymes was analyzed (Fig. 4) the main effect of stress was detected for GSH-Red [$F(1,12) = 39.65$; $P < 0.0001$], SOD2 [$F(1,12) = 13.77$; $P < 0.01$] and CAT [$F(1,12) = 7.66$; $P < 0.05$]. The main effect of fructose was observed for GSH-Red [$F(1,12) = 35.65$; $P < 0.0001$], SOD2 [$F(1,12) = 14.98$; $P < 0.01$] and CAT [$F(1,12) = 11.01$; $P < 0.01$], while significant interaction between factors was found for GSH-Red [$F(1,12) = 18.93$; $P < 0.001$] and SOD2 [$F(1,12) = 9.25$; $P < 0.05$]. Furthermore, protein level of GSH-Red and SOD2 was reduced in the hypothalamus of all treated groups in comparison to the control ($P < 0.001$ for GSH-Red, and $P < 0.01$ for SOD2), while CAT was reduced in stressed fructose-fed animals compared to the controls ($P < 0.01$) as well as to fructose-fed and to stressed group on standard diet ($P < 0.05$).

The protein level of NFκB and its inhibitor IκB was not altered in any of the experimental groups (Fig. 5A), while significant effect of stress on IL-1β [$F(1,19) = 9.18$; $P < 0.01$], IL-6 [$F(1,20) = 4.54$; $P < 0.05$] and TNFα [$F(1,16) = 14.95$; $P < 0.01$] as well as the interaction of stress and fructose on TNFα [$F(1,16) = 4.67$; $P < 0.05$] was observed. Post-hoc test indicated diminished IL-1β, IL-6 and TNFα expression in stressed fructose-fed females compared to untreated animals ($P < 0.05$), and to unstressed fructose-fed animals ($P < 0.05$ for IL-1β and $P < 0.01$ for TNFα) (Fig. 5B).

Discussion

The main results of this study show that chronic exposure to stress combined with fructose-enriched diet reduced insulin and elevated glucocorticoid signaling in the hypothalamus of adult female rats, while the alterations in the appetite regulation were not observed. In addition, antioxidative defense was compromised.

Disturbed hypothalamic insulin signaling has been reported in male rats after chronic stress, as well as after fructose overconsumption [2, 3]. However, in our study done on females, combination of these factors was necessary to elicit such effect. Namely, nine-week consumption of fructose together with the exposure to unpredictable stress decreased total Akt and both phosphorylated forms – pAkt-Ser^{473} and pAkt-Thr^{308} in the hypothalamus of female rats. Considering that depletion of Akt prominently inhibits the insulin responsiveness [31], our results indicate decreased Akt activity in our experimental paradigm although the ratio of phosphorylated form to total Akt was not altered. This suggests reduced hypothalamic insulin signaling. Similar findings were obtained by Battu [32] who reported significant decrease in pAkt-Ser^{473} and unaltered total IRS1 and pIRS1-Ser^{307} after 4 months of diet rich in simple

sugars and saturated fat, and by Zhang [2] who observed disrupted insulin signaling evidenced by decreased phosphorylation of insulin receptor and Akt in the hypothalamus after 4 weeks of high-fructose diet. Inhibition of hypothalamic insulin signaling has also been observed in rats exposed to chronic unpredictable stress for 8 weeks [3], and even after short-term exposure to cold [33].

Glucocorticoid hormones have the ability to downregulate insulin signaling in the brain. Namely, prolonged dexamethasone administration attenuated insulin signaling in rat hypothalamus, affecting pAkt-Ser^{473} among other components [5]. Our results suggest activation of glucocorticoid pathway in fructose-fed stressed animals based on increased protein level of GR as well as of both enzymes responsible for the intracellular glucocorticoid regeneration (HSD1 and H6PDH). It can be postulated that increased glucocorticoid signaling could disturb insulin signaling in the hypothalamus, although this needs to be further examined. It should be kept in mind that insulin is a major inhibitor of HSD1 [34], and although the sequence of events is not known, disturbed insulin signaling contributes to the HSD1 overexpression and vice versa.

As some people lose appetite in response to stress conditions while others reach for comfort food [9], decreased body and visceral adipose tissue mass in stressed females that we observed in our previously published data obtained on the same experimental animals [35] was expected to be a consequence of reduced appetite due to reduced expression of orexigenic neuropeptides and/or elevated expression of anorexigenic neuropeptides in the hypothalamus. However, the unchanged expression of AgRP, NPY, POMC and CART was consistent with similar energy intake of all experimental groups in our study. As insulin and glucocorticoids coordinate expression of these genes [9-12], it was surprising that impaired insulin and elevated glucocorticoid signaling in stressed animals on fructose diet did not increase appetite and visceral adipose tissue mass. Nevertheless, desensitization of hypothalamic insulin signaling is not necessarily accompanied with obesity [5].

Although stress has been mainly shown to elevate expression of anorexigenic neuropeptides [12], Sefton and colleagues [36] have shown that chronic corticosterone treatment does not affect POMC and CART mRNA level in the hypothalamus. It seems that the direction and intensity of NPY expression is largely dependent on stress type and duration. While stimulatory effects of acute [37], and inhibitory effect of chronic stress [38] have been generally reported, some studies [39, 40], including the one presented herein, indicate that stress does not affect the expression of orexigenic neuropeptides even though it reduces body weight. Considering this, glucocorticoid hormones, as mediators of stress response, do not necessarily affect appetite to change fat mass. Thus, the observed visceral adipose tissue loss in both stressed groups may be a consequence of increased fatty acid oxidation in the tissue itself (results previously published [35]).

Leptin is another important regulator of orexigenic and anorexigenic neuropeptides in the hypothalamus. Plasma leptin is directly proportional to adipose tissue mass [13] and our results reflect this correlation since stressed animals have been reported to have significantly reduced visceral adipose tissue mass [35] and plasma leptin regardless of the diet. The ability of stress to reduce plasma leptin level without increasing appetite was previously demonstrated [40]. Even a stress-induced increase in leptin and leptin receptor levels does not necessarily affect the NPY expression [41]. Our study contributes to these findings suggesting that control of food intake under stress conditions is not mediated by leptin, even when fructose-enriched diet is consumed.

While some studies reported fructose-induced hyperleptinemia [14], others did not [42]. Also, hyperleptinemia has been associated with leptin resistance [13]. In the present study, leptin signaling at the level of plasma leptin, ObRb, and SOCS3 was not affected by fructose consumption.

Of note are recent human studies, demonstrating that fructose can activate other brain regions, like those involved in attention and reward response or in cognitive functions. Namely, in the study by Luo et al. [43] fructose, compared to the equivalent dose of glucose, resulted in greater brain reactivity of visual cortex to food cues as well as in a greater appetite, desire for food and preference for immediate food-related rewards promoting feeding behavior. In addition, Zanchi el al. [44] observed increased functional connectivity in networks related to cognitive functions after acute fructose intake.

Metabolic perturbations including diabetes have been related to hypothalamic oxidative stress in several models. Namely, oxidative damage in the hypothalamus has been strongly associated with diabetes in IRS2 knock-out mice [18], while increased hypothalamic lipid peroxidation, and reduced GSH-Px and glutathione levels indicated elevated oxidative stress and diminished antioxidative defense in the hypothalamus of streptozotocin-treated diabetic rats [45]. On the other hand, suppression of hypothalamic oxidative stress improved insulin resistance [20], and antioxidant treatment of mouse hypothalamic neurons after the induction of endoplasmic reticulum stress recovered the decrease of Akt phosphorylation [22]. Both fructose and stress may elicit oxidative stress. High-fructose diet has been shown to disturb cellular antioxidative defense system and enhanced plasma lipid peroxidation in rats [21], which has been alleviated after treatment with insulin sensitizer metformin. Also, chronic cold stress has been shown to reduce CAT, GSH-Px and GSH-Red activities and to deplete total antioxidative capacity in the hypothalamus [46]. In the present study, only combined application of stress and fructose diet significantly reduced protein levels of antioxidative enzymes (SOD2, GSH-Red and CAT), indicating reduced antioxidative capacity in the hypothalamus. This change was parallel with perturbations in insulin signaling in this brain region. It has been documented that prolonged exposure to exogenous glucocorticoids provoked cellular oxidative stress [47], and that subcutaneous corticosterone

administration induced generation of reactive oxygen species and decreased activity of antioxidative enzymes in the hippocampus [48]. In line with this, locally elevated glucocorticoid signaling in the hypothalamus might underlie decreased antioxidative defense in stressed animals on fructose diet in the present study.

Regardless of the cause, reduced insulin signaling and antioxidative protection indicate that combination of prolonged stress and fructose diet disturbs hypothalamic regulation of metabolic homeostasis. Unbalanced reactive oxygen species production leads to formation of lipid peroxides, endoplasmic reticulum stress, and loss of DNA integrity, disturbing cell function and finally inducing apoptosis. Activation of insulin signaling components including Akt mediate antiapoptotic effect of insulin [49]. Thus, beside the possibility that oxidative stress might disturb hypothalamic insulin signaling, the deficiency of Akt-mediated signals could further augment oxidative damage.

Metabolic disorders are not only associated with systemic low-grade inflammation, but also with hypothalamic inflammation, which disrupts regulation of energy homeostasis (Reviewed in [23]). Data on stress-related perturbations in hypothalamic inflammatory status are rather conflicting, as the expression of proinflammatory cytokines has been reported to be elevated [27], or unchanged [28] in male rats after chronic stress. As for nutrients, high fat diet has been predominantly described to induce obesity-related hypothalamic inflammation [50], though, fructose overconsumption also has a potential to activate NFκB signaling pathway [2, 26]. However, most *in vivo* studies describing fructose-related neuro-inflammation, also reported increased fat mass and disturbed lipid status in the form of elevated plasma triglycerides and free fatty acids. Although fructose has been proposed to potentiate hepatic production of triglycerides, this lipogenic effect has not been observed in our previous study on female rats [35]. Interestingly, reduced expression of proinflammatory cytokines in the hypothalamus of stressed female rats on fructose diet indicate reduced inflammatory response, which can make these animals prone to infections. In the study of Marissal-Arvy et al. [51] acute restraint stress reduced hypothalamic expression of proinflammatory cytokines in male rats while high fat/high fructose diet abolished this adverse effect. Considering this, it seems that lipid dietary components are important in eliciting hypothalamic inflammation. As reviewed in [52], saturated fatty acids and their metabolites can trigger proinflammatory pathways, and long-chain species have the ability to directly act in the brain as they accumulate within the hypothalamus during high-fat diet regime.

Direct interaction of GR with p65 in the nucleus inhibits NFκB-mediated expression of proinflammatory genes [53]. Since the protein level of NFκB and its inhibitor IκB were not altered, we propose that glucocorticoid hormones are responsible for the reduction of proinflammatory cytokines

based on the observation that GR and both enzymes involved in the regeneration of active glucocorticoids were elevated in stressed female rats on fructose diet.

In conclusion, our results point that stress exposure combined with fructose overconsumption for a prolonged time has more detrimental effects on hypothalamic function than stress or fructose-enriched diet applied separately. The combined treatment enhanced glucocorticoid signaling, and impaired insulin signaling, antioxidative defense and inflammatory reaction of this homeostasis-regulating center.

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Statement of Ethics

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade. Permit number: 02-11/14.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Study conception and design: IE, GM; acquisition of data: SK; analysis and interpretation of data: IE, SK, JN; Writing of the article: IE; revising for important intellectual content GM, JN, SK; Final approval of the version to be submitted IE, JN, GM, SK.

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600

601 **Figure Captions**

602

603 **Fig. 1. Hypothalamic insulin signaling.** The level of total IRS1, pIRS1-Ser^{307}, total Akt, pAkt-Ser^{473} and
604 pAkt-Thr^{308} proteins were measured by Western blot in the tissue extracts obtained from the whole

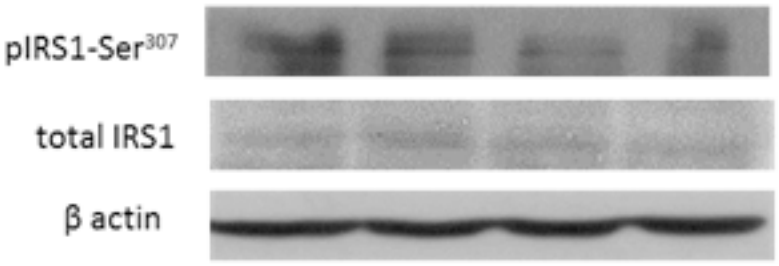
hypothalamus from control (C), fructose (F), stress (S) and stress + fructose (SF) group of animals. Bar graphs represent the means ± SEM for each protein normalized to β actin expressed in arbitrary units (AU) as well as for the ratio of phosphorylated and total protein. Statistical significance of the difference between experimental groups (two-way ANOVA): ***P < 0.001, SF versus C; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001, SF versus F; $^{\$\$}$P < 0.01 and $^{\$\$\$}$P < 0.001, SF versus S. IRS1 – insulin receptor substrate 1; pIRS1-Ser^{307} – phosphorylation of IRS1 on Ser307; Akt – protein kinase B; pAkt-Ser^{473} – phosphorylation of Akt on Ser473; pAkt-Thr^{308} – phosphorylation of Akt on Thr308.

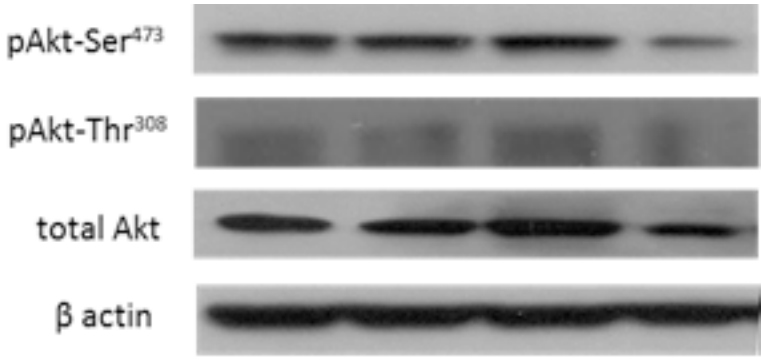
Fig. 2. Hypothalamic glucocorticoid signaling. Protein levels of HSD1, H6PDH and GR were measured by Western blot in the tissue extracts obtained from the whole hypothalamus from control (C), fructose (F), stress (S) and stress + fructose (SF) group of animals, normalized to β actin and expressed in arbitrary units (AU). The values represent the means ± SEM. Statistical significance of the difference between experimental groups (two-way ANOVA): *P < 0.05 and **P < 0.01, SF versus C; $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01, SF versus F; $^{\$}$P < 0.05 and $^{\$\$}$P < 0.01, SF versus S. HSD1 – 11β-hydroxysteroid dehydrogenase type 1; H6PDH – hexose-6-phosphate dehydrogenase; GR – glucocorticoid receptor.

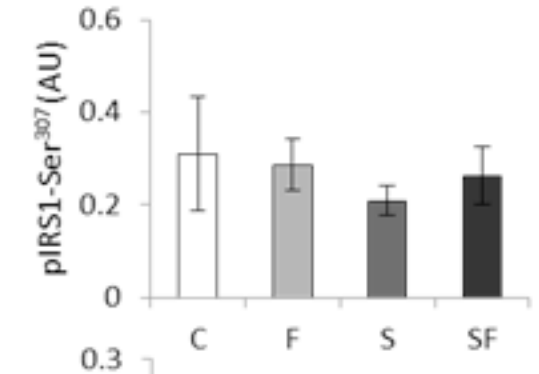
Fig. 3. Leptin signaling and the expression of orexigenic and anorexigenic neuropeptides. Groups: control (C), fructose (F), stress (S) and stress + fructose (SF) The protein level of ObRb and SOCS3 (normalized to β actin) was measured by Western blot in the tissue extracts obtained from the whole hypothalamus. TaqMan real-time PCR was used to determine the level of ObRb, SOCS3, NPY and AgRP mRNAs relative to HPRT mRNA, while SYBR® Green real-time PCR was used to determine the level of POMC and CART mRNAs relative to HPRT mRNA. The values represent the mean ± SEM. Statistical significance of the difference between experimental groups (two-way ANOVA): *P < 0.05 and ***P < 0.001, S or SF versus C; $^{\#}$P < 0.05 and $^{\#\#\#}$P < 0.001, SF versus F; $^{\$}$P < 0.05 and $^{\$\$}$P < 0.01, SF versus S. ObRb – leptin receptor; SOCS3 – suppressor of cytokine signaling 3; HPRT – hypoxanthine phosphoribosyl transferase 1; NPY – neuropeptide Y; AgRP – agouti-related protein; POMC – proopiomelanocortin; CART – cocaine and amphetamine-regulated transcript.

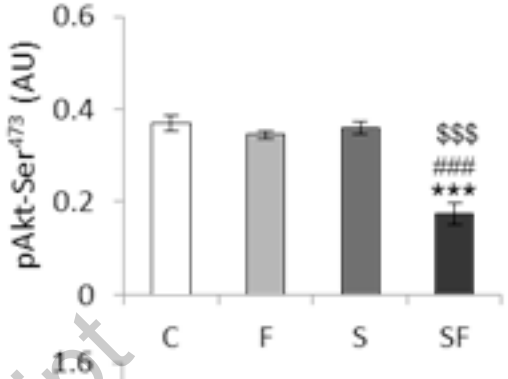
Fig. 4. Hypothalamic level of antioxidative enzymes. Protein levels of SOD1, SOD2, CAT GSH-Px, and GSH-Red were measured by Western blot in the tissue extracts obtained from the whole hypothalamus from control (C), fructose (F), stress (S) and stress + fructose (SF) group of animals, normalized to GAPDH and expressed in arbitrary units (AU). The values represent the means ± SEM. Statistical significance of the difference between experimental groups (two-way ANOVA): **P < 0.01

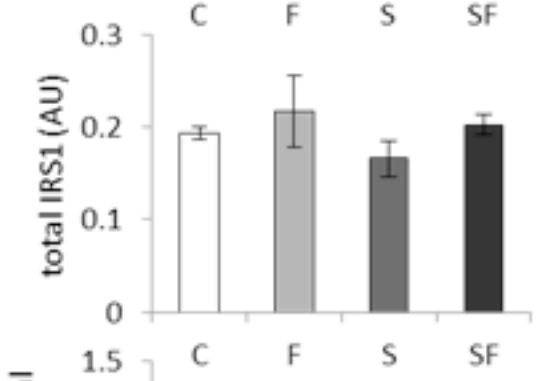
638 and ***P < 0.001 – F, S or SF versus C; #P < 0.05, SF versus F and $P < 0.05, SF versus S. SOD1 –
639 cytoplasmic copper-zinc superoxide dismutase 1; SOD2 – mitochondrial manganese superoxide dismutase
640 2; CAT – catalase; GSH-Px – glutathione peroxidase; GSH-Red – glutathione reductase; GAPDH –
641 glyceraldehydes-3-phosphate dehydrogenase.
642
643 **Fig. 5. Hypothalamic inflammatory status.** Groups: control (C), fructose (F), stress (S) and stress +
644 fructose (SF) **A**. The protein levels of NFκB and IκB (normalized to β actin) were measured by Western
645 blot in the tissue extracts obtained from the whole hypothalamus. The values represent the means ± SEM.
646 **B**. The level of IL-1β, IL-6 and TNFα mRNAs relative to HPRT mRNA were determined by TaqMan
647 real-time PCR in the hypothalamus. The values represent the mean ± SEM. All measurements were done
648 in triplicate. Statistical significance of the difference between experimental groups (two-way ANOVA):
649 *P < 0.05, SF versus C; #P < 0.05 and, ##P < 0.01, SF versus F. NFκB – nuclear factor-κB; HPRT –
650 hypoxanthine phosphoribosyl transferase 1.

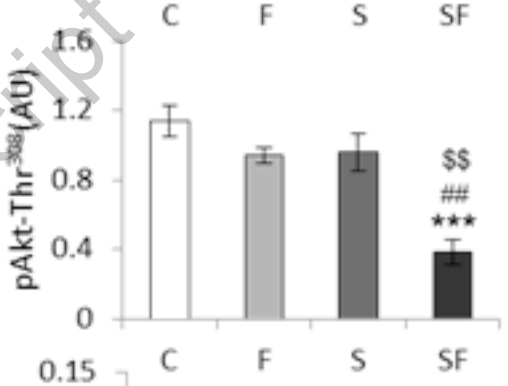


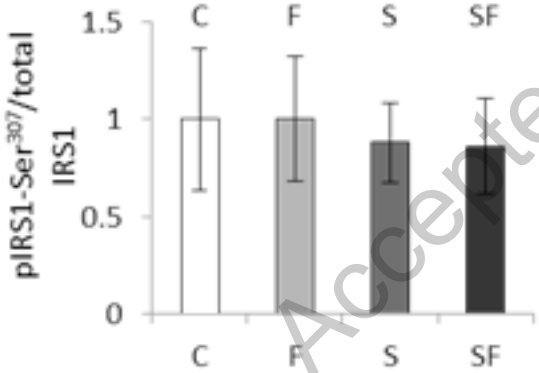


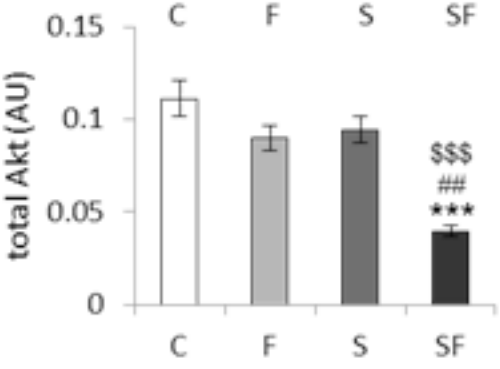


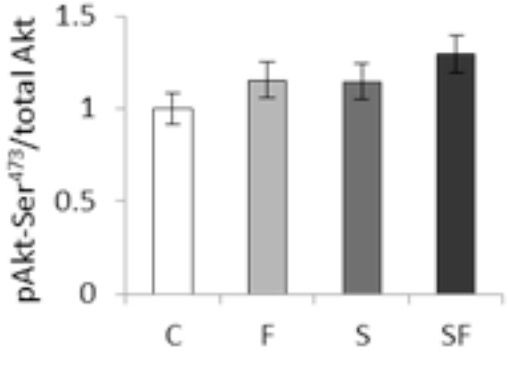


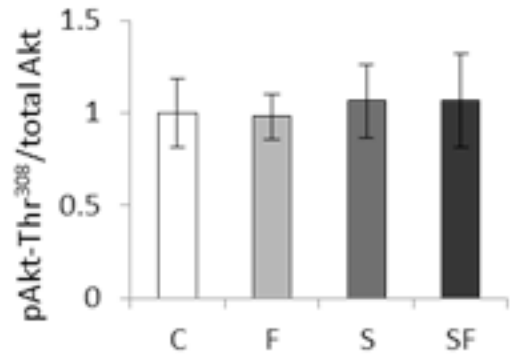


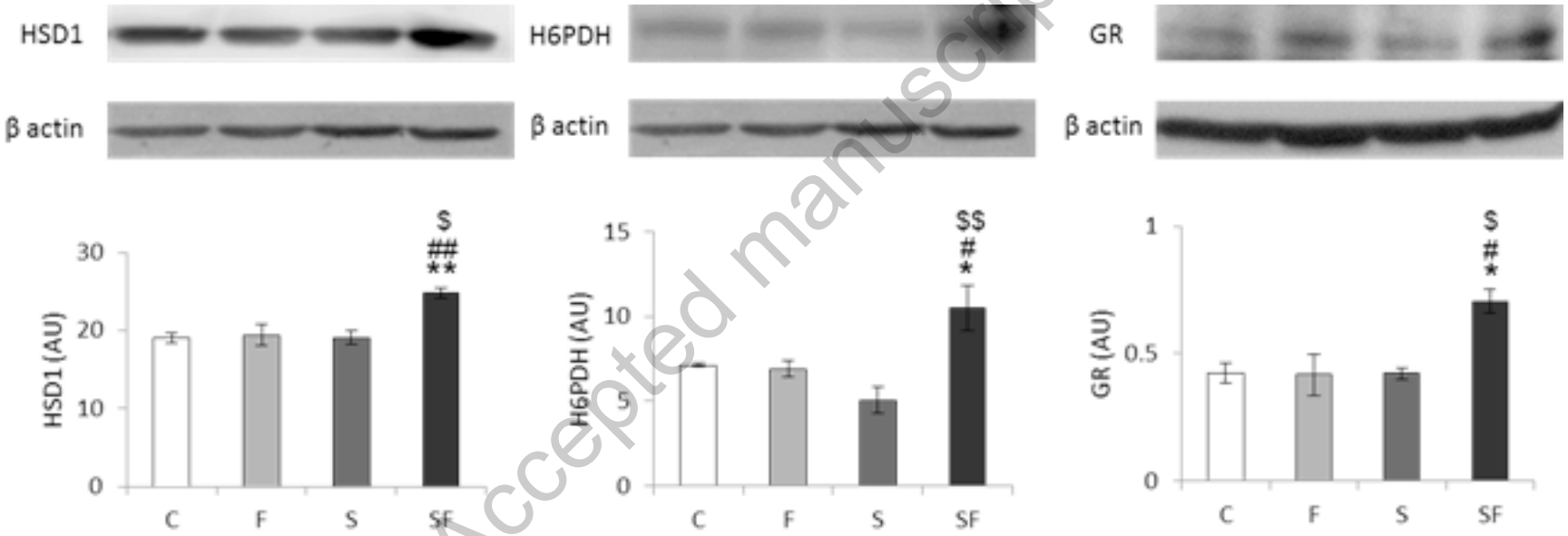


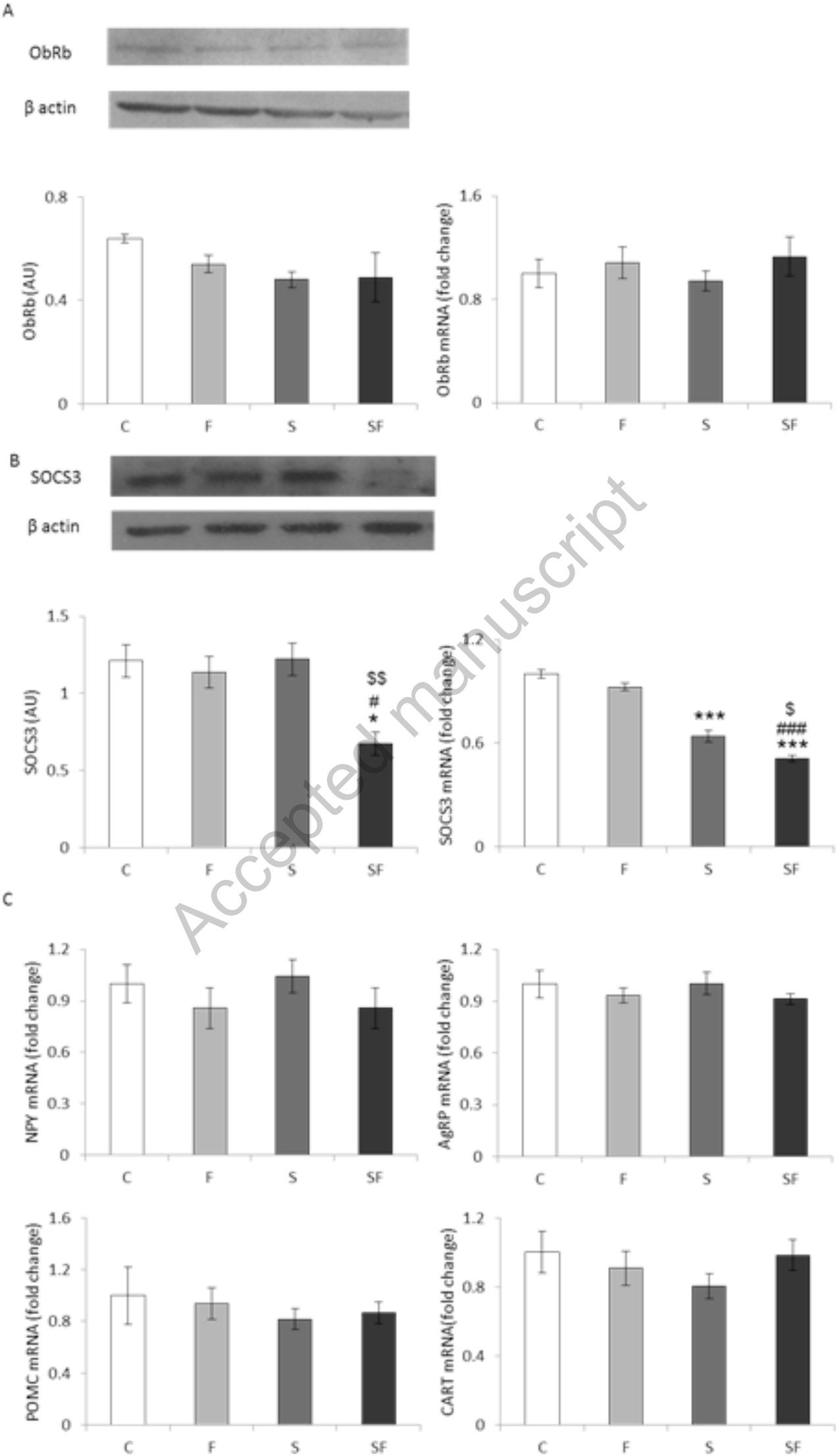


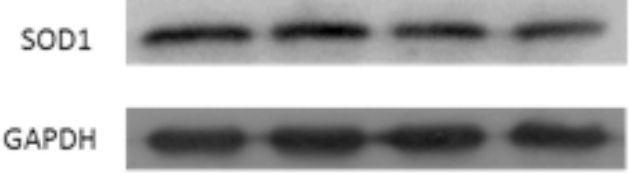


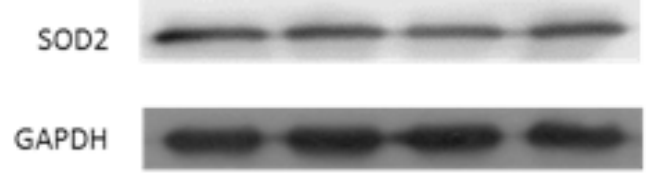


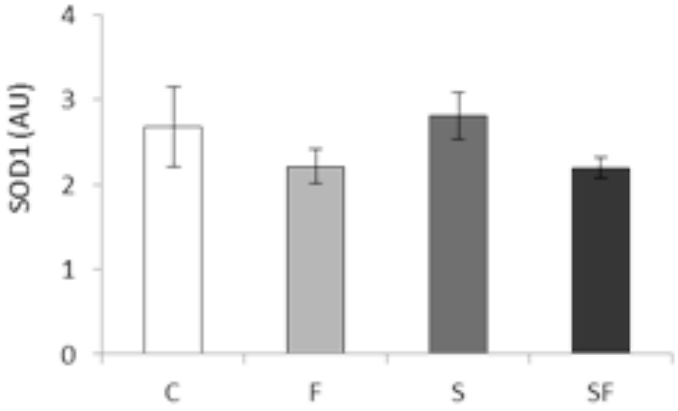


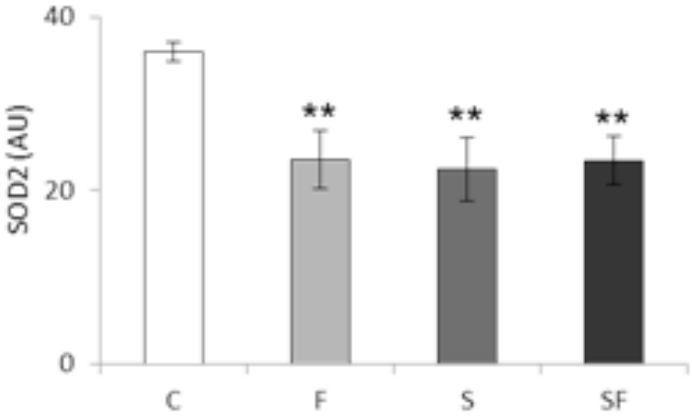


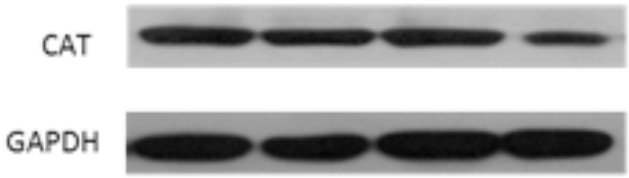


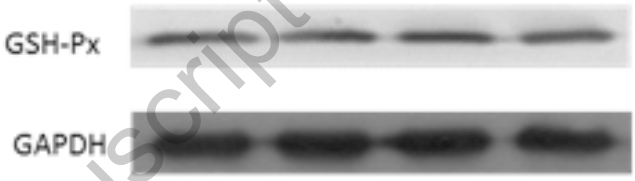


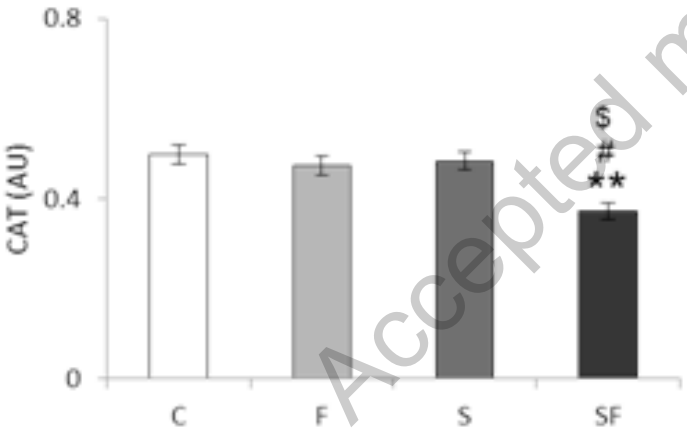


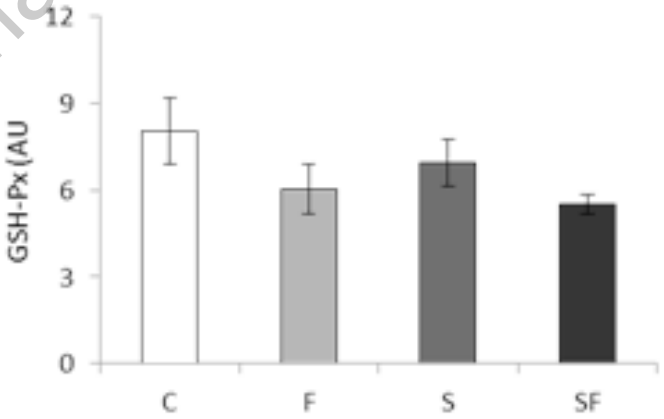


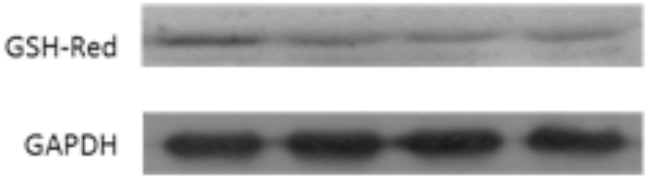


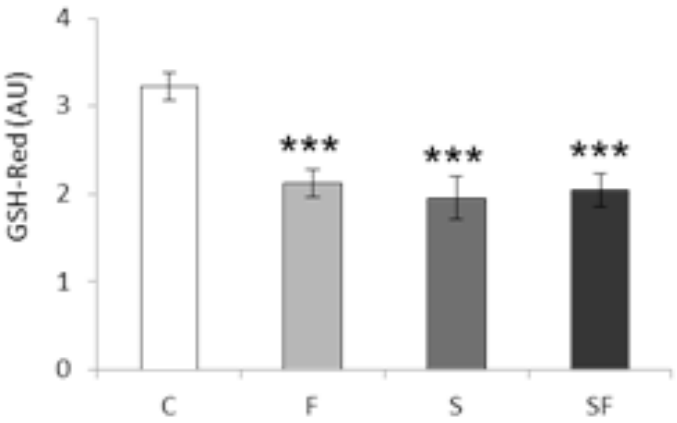












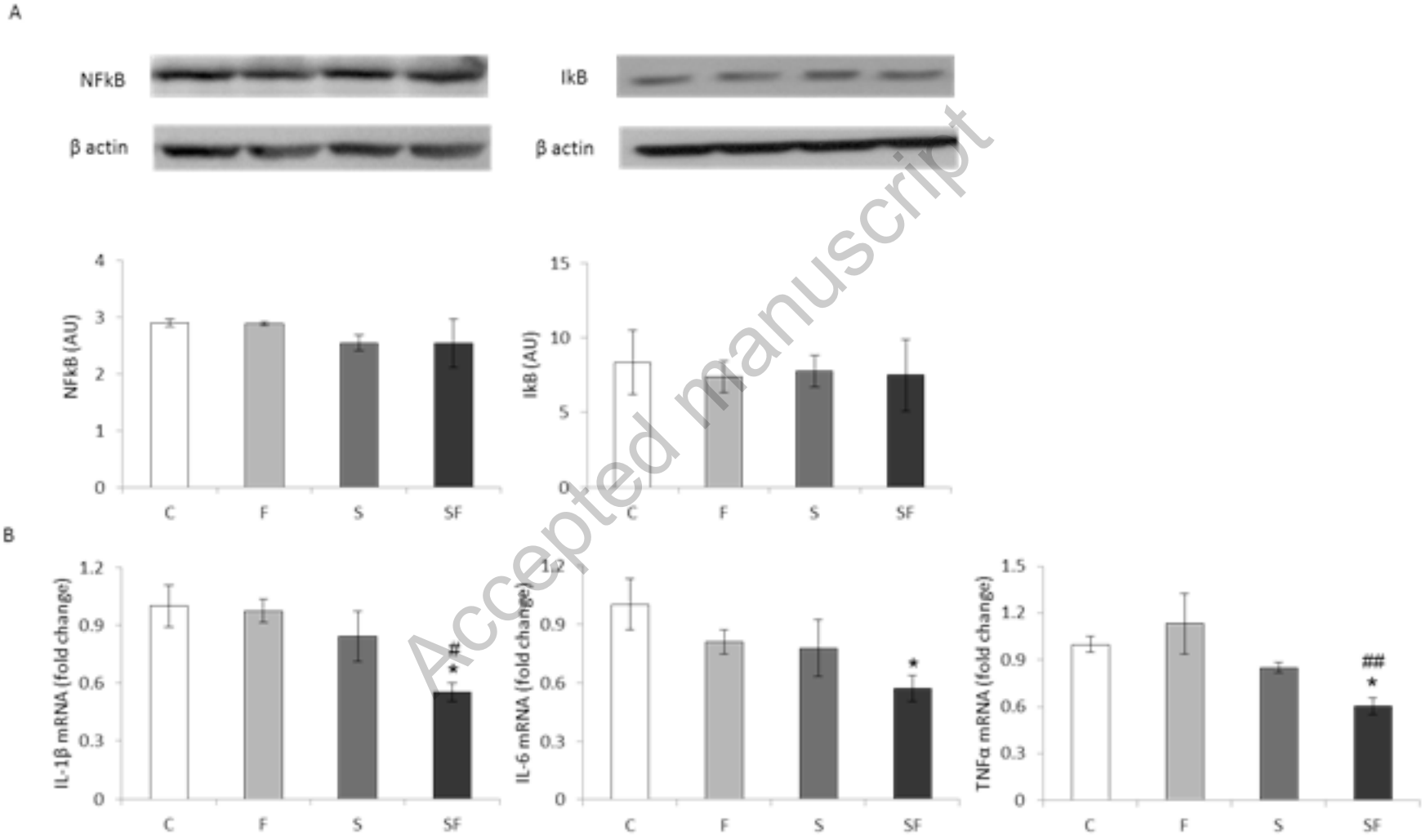


Table 1. Energy intake and plasma leptin concentration

	Control	**Fructose**	**Stress**	**Stress+ Fructose**
Energy intake (kJ)	259.66 ± 6.87	301.22 ± 14.42	253.04 ± 3.98	284.97 ± 15.50
Leptin (ng/mL)	1.87 ± 0.19	1.85 ± 0.21	0.88 ± 0.17**	1.04 ± 0.14* #

Values are expressed as mean ± SEM;

Energy intake is expressed per day per animal;

* $P < 0.05$, ** $P < 0.01$, between treated animals and control group

# $P < 0.05$, between stressed and unstressed fructose-fed animals