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Combination of chronic stress with fructose diet increases AMP-activated protein kinase phosphorylation and affects agouti-related protein and proopiomelanocortin expression in the hypothalamus of male Wistar rats

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Appetite regulation in the hypothalamus is dependent on hormonal signals from the periphery, such as insulin and leptin, and can be modulated by both sugar-rich diet and stress. Our aim was to explore the effects of 9-week feeding with 20% fructose solution combined with 4-week chronic unpredictable stress, on appetite-regulating neuropeptides and modulatory role of leptin and insulin signalling in the hypothalamus of male Wistar rats. Energy intake, body mass and adiposity, as well as circulatory leptin and insulin concentrations were assessed. Hypothalamic insulin signalling was analysed at the level of glucose transporters, as well as at the protein level and phosphorylation of insulin receptor, insulin receptor supstrate-1, Akt and ERK. Phosphorylation of AMP-activated protein kinase (AMPK), level of protein tyrosine phosphatase 1B (PTP1B) and expression of leptin receptor (ObRb) and suppressor of cytokine signalling 3 (SOCS3) were also analysed, together with the expression of orexigenic agouti-related protein (AgRP) and anorexigenic proopiomelanocortin (POMC) neuropeptides. The results revealed that stress decreased body mass and adiposity, blood leptin level and expression of ObRb, SOCS3 and POMC, while combination with fructose diet led to marked increase of AgRP, associated with AMPK phosphorylation despite increased plasma insulin. Reduced Akt, enhanced ERK activity and elevated PTP1B were also observed in the hypothalamus of these animals. In conclusion, our results showed that joint effects of fructose diet and stress are more deleterious than the separate ones, since inappropriate appetite control in the hypothalamus may provide a setting for the disturbed energy homeostasis in the long run.

Key words: fructose diet; chronic stress; hypothalamus; AMPK; appetite-regulating neuropeptides

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Abbreviations: AgRP, agouti-related protein; AMPK, AMP-activated protein kinase; CART, cocaine- and amphetamine-regulated transcript; IR, insulin receptor; IRS, insulin receptor substrate; NPY, neuropeptide Y; SOCS3, suppressor of cytokine signalling; POMC, proopiomelanocortin; PTP1B, protein tyrosine phosphatase 1B; ObRb, leptin receptor; VAT, visceral adipose tissue

INTRODUCTION

Hypothalamus is the brain structure responsible for the regulation of food intake and appetite mainly through the control of orexigenic and anorexigenic neuropeptides. Excessive consumption of sugars is among the factors that can disturb appetite regulation in the hypothalamus, ultimately leading to obesity, cardiovascular and neurological disorders (Samodien *et al.*, 2019). Dramatic increase in fructose consumption, originating mainly from consumption of palatable fructose sweetened beverages, has been related to the higher prevalence of type 2 diabetes and obesity in modern society (Bray *et al.*, 2004; Basciano *et al.*, 2005). However, another important factor that correlates with the increasing emergence of these pathologies is everyday chronic stress (Kyrou & Tsigos, 2007). Mechanisms by which stress can modulate sugar-related eating behaviour are still poorly understood, since some individuals respond to stress with food avoidance, while others crave for sugar and fat abundant food (Dallman *et al.*, 2003). It has been suggested that chronic stress can dysregulate the hypothalamus-adipose tissue axis over time, which consequently affects glucose metabolism, promotes insulin resistance and influences multiple appetite-related hormones in the hypothalamus (Deck *et al.*, 2017).

The most prominent early hallmark of the dysfunction of hypothalamus-adipose tissue axis is the onset of leptin resistance, which is often associated with the accumulation of visceral fat, elevated plasma leptin concentrations and, most importantly, with failure to respond to the hormone at central and peripheral levels (Munzberg & Myers Jr., 2005). Apart from leptin, disruption of glucose homeostasis and brain insulin signalling can also induce hyperphagia and obesity in rodents (Thon *et al.*, 2016). Glucose represents a major substrate for brain energy production and its transportation is conducted via facilitative glucose transporters (GLUTs) among which GLUT1 is primarily involved in glucose transportation through the blood-brain barrier, GLUT2 is a key mediator of cellular glucose sensing and GLUT3 controls the glucose uptake into the neurons (Navale & Paranjape, 2016). Insulin, as the key regulator of glucose uptake, activates insulin receptor (IR), which further phosphorylates insulin receptor substrates (IRSs), thus activating downward signalling pathways, regulated by phosphatidylinositol-3-kinase (PI3K)/Akt and MAPK/ERK1/2 (Riehle & Abel, 2016). Chronic activation of MEK1-

ERK or MKK6/3-p38 pathway leads to increased expression of GLUT1 and downregulation of expression of GLUT4, which results in increased basal glucose transport, but decreased transport induced by insulin (Fujishiro *et al.*, 2003). Apart from this, ERKs have been described as mediators of increased glucose usage induced by leptin, as well as of regulators of whole body insulin sensitivity (Toda *et al.*, 2013). It has been previously described that both excessive fructose consumption and exposure to chronic stress, reduce phosphorylation of insulin receptor at Tyr-1162/1163 (pIRBTyr$^{1162/1163}$), as well as phosphorylation of protein kinase B (Akt) at Ser-473 (pAktSer^{473}) in the hypothalamus, thus promoting insulin resistance (Zhang *et al.*, 2014; Pan *et al.*, 2013). Several other mechanisms may potentially contribute to the central insulin and leptin resistance, including defective hormone transport across the blood–brain barrier, downregulation of their receptors, or induction of inhibitors like suppressor of cytokine signalling (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) (Myers *et al.*, 2008).

The crosstalk between at least two adiposity signals, insulin and leptin, can play a significant role in the central regulation of appetite (Hillebrand & Geary, 2010), since these hormones bind to their specific receptors, IR and leptin receptor (ObRb), and tightly regulate orexigenic and anorexigenic peptides synthesis and secretion within the arcuate nucleus of the hypothalamus. Namely, leptin and insulin inhibit neuropeptide Y (NPY)/agouti-related protein (AgRP) and stimulate proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neuronal activity, resulting in lower food intake and higher energy expenditure (Korner *et al.*, 2001; Morton *et al.*, 2006). AMP-activated protein kinase (AMPK) in the hypothalamus acts as the integrator of nutritional and hormonal signals (De Morentin *et al.*, 2011). Among different factors that can influence energy intake and expenditure through hypothalamic AMPK, insulin and leptin are considered as inhibitory and anorexigenic, while ghrelin, glucocorticoids or cannabinoids are considered as AMPK activators generally capable to upregulate the expression of orexigenic neuropeptides (Huynh *et al.*, 2016).

Previous studies, including our own on female rats, have shown that insulin and leptin actions in the hypothalamus could be crucial for the regulation of energy intake and whole body energy balance (Kovacevic *et al.*, 2019; Lowette *et al.*, 2015). With this in mind, in this study on male rats, we investigated the combined effects of diet enriched with 20% fructose solution and chronic unpredictable stress on hypothalamic insulin and leptin signalling and their effect energy sensing and appetite control.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats, 2.5 months old were bred in our laboratory and divided randomly into four experimental groups (n=9 animals per group): C – control group was kept on standard diet (commercial food and tap water); C+F – fructose-fed control group ate the same food and drank fructose solution (20% w/v) (API-Pek Bečej, Serbia) instead of tap water; S – stress group had standard diet and was exposed to chronic unpredictable stress, in the last 4 weeks of the 9-week dietary treatment and finally, S+F – fructose-fed stressed group that had the diet regime of the C+F group and was subjected to stress like the S group. Duration, type and sequence of stressors and concentration of fructose solution were selected in the way to bear a resemblance to lifestyle of modern humans (Joels *et al.*, 2004; Ventura *et al.*, 2011). The composition of the rodent food was given in detail previously (Teofilovic *et al.*, 2020). The stress protocol was modified from Joels *et al.* (2004) and consisted of daily-applied stressors: 30 min physical restraint, 50 min cold room (4°C) exposure, 10 min forced swimming in cold water, 60 min of cage rocking, 4 h keeping on wet bedding and overnight keeping in tilted cages (45°C). Both type and number of daily stressors, as well as the time of stress exposure (between 9 a.m. and 4 p.m. for all the stressors except for the cage tilt) were selected randomly. None of the stressors were applied consecutively or twice per day. Three animals were kept in one cage in room with controlled temperature (22±2°C) and 12 h light/dark cycle (lights on at 7 a.m.). All experimental groups had access to food and fluids ad libitum. Food and fluid intake were measured every day, while body mass was recorded every week. Energy intake for rats on standard diet was calculated as food weight (g)×11 kJ, while energy intake for rats on fructose diet was calculated as sum of calories ingested as food and fructose solution [food weight (g)×11 kJ+fructose intake (ml)×3.44 kJ]. The animal procedures gained approval from the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (Permit No. 3-12/12). All procedures were complied with the EEC Directive 2010/63/EU.

Determination of physiological and biochemical parameters

After the end of the treatment, animals were subjected to fasting overnight and killed rapidly by decapitation with a guillotine (Harvard Apparatus, Holliston, MA, USA). VAT (retroperitoneal and perirenal depots of white adipose tissue) was immediately excised, washed in saline, dried and weighed. EDTA containing tubes were used for trunk blood collection. Low speed centrifugation (1600×*g*/10 min) was used for plasma preparation, which was stored at −20°C for further processing.

Corticosterone concentrations in plasma were assessed by Corticosterone EIA kit according to manufacturer's instructions (Immunodiagnostic Systems LTD, East Boldon, UK). Absorbance at 450 nm (reference 650 nm) was measured spectrophotometrically on Multiskan Spectrum (Thermo Fisher Scientific, Waltham, MA, USA) with assay sensitivity of 0.17 ng/ml. Corticosterone concentrations were calculated using 4PL curve fitting method (Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA). Intra-assay and inter-assay CVs were 5.9% and 8.9%, respectively.

Commercial RIA method (INEP, Belgrade, Serbia) was used to determine insulin concentrations in plasma. The assay sensitivity was 0.6 mIU/l and intra-assay coefficient of variation (CV) was 5.24%.

Leptin in plasma was measured by Rat Leptin ELISA kit (Merck Millipore, Burlington, MA, USA) according to the instructions from the manufacturer. The assay sensitivity was 0.04 ng/ml. Intra-assay coefficient of variation (CV) was 1.88%, while inter-assay CV was 3.31%.

RNA extraction and reverse transcription

The hypothalamus was removed from the ventral side of the brain (having the optic chiasm as rostral, thalamus

as dorsal and mammillary bodies as caudal limit), rapidly frozen and kept in liquid nitrogen.

TRIzol® (AmBion, Life Technologies, Carlsbad, USA) was used for the isolation of total RNA. Samples were centrifuged at 12000×*g* for 15 min at 4°C after homogenization. RNA was precipitated with isopropanol. Quantitative and qualitative evaluation of the isolated RNA was performed spectrophotometrically (OD 260/280>1.8 was considered satisfactory) and on 2% agarose gel with ethidium bromide. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to manufacturer's instructions and cDNAs were stored at −70°C. Prior to the cDNA synthesis, DNA was removed by DNA-ase I treatment.

Real-time PCR

TaqMan® Gene Expression Assay primer-probe mix were used for the detection of AgRP (Rn01431703_g1), ObRb (Rn00561369_m1), SOCS3 (Rn00585674_s1) and hypoxanthine phosphoribosyl transferase 1 (HPRT1) (Rn01527840_m1). The expression of POMC was determined using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primer pairs: POMC: F 5'-TCCATAGACGTGTGGAGCTG-3', R 5'-GACGTACTTCCGGGGATTTT-3'; HPRT1: F 5'-CAGTCCCAGCGTCGTGATTA-3', R 5'-AGCAAGTCTTTCAGTCCTGTC-3' (Invitrogen, Carlsbad, CA, USA). PCR was performed using Quant Studio™ 3 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µl containing 20 ng of cDNA template. Conditions were as follows: 2 min at 50°C, 10 min at 95°C, 15 sec at 95°C (40 cycles) and 60 sec at 60°C. No template control was included in every run. To confirm the single PCR product, melting curve analyses were performed for SYBR® Green. Relative quantification was performed using the comparative 2-ΔΔCt method. Results analysis was performed in the Quant Studio™ Design and Analysis Software v1.4.0 (Applied Biosystems, Foster City, USA) with a confidence level of 95% ($p \leq 0.05$).

Preparation of total protein

The organic phase was used to obtain total protein fraction. Total proteins were precipitated from the phenol-ethanol supernatant after addition of acetone and subsequent centrifugation for 10 min at 12000×*g* (4°C). The obtained protein pellet was sonicated on ice in 0.3 M guanidine hydrochloride in 95% ethanol and 2.5% glycerol and subsequently washed three times in the same buffer. After protein pelleting by 5 min centrifugation at 8000×*g* (4°C), pellets were resuspended in the lysis buffer with 2.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 50 mM DTT and stored at –70°C until further use.

Western blot analysis

Protein concentration was determined using the Lowry method (Lowry *et al*., 1951). Boiled samples (2×Laemmli's buffer for 5 min) were separated using electrophoresis on 7.5% SDS PAG (50 µg of proteins per lane). Proteins were transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Burlington, MA, USA). Membranes were blocked (5% BSA or 3% non-fat dry milk) and subsequently incubated with appropriate primary antibodies at 4°C overnight: anti-pAkt-Ser473 (sc-7985-R; 1:1000), anti-Akt (sc-8312; 1:1000), anti-pIRB (sc-25103; 1:1000), anti-IRB (sc-711; 1:500), anti-pIRS1-Ser307(sc-33956; 1:1000) and anti-PTP1B (sc-1718-R; 1:500) anti-bodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-β actin antibody (ab8227; 1:10000), anti-GLUT1 (ab625; 1:10000), anti-GLUT2 (ab95256; 1:1000) and anti-GLUT3 (ab41525; 1:4000) antibodies were from Abcam (Cambridge, UK), while ERK (91025; 1:1000), pERK Thr202/Tyr204 (9101s; 1:1000) and pAMPK Thr172 (#41885, 1:1000) were purchased from Cell Signalling (Danvers, MA, USA). Secondary anti-rabbit IgG horseradish peroxidase (HRP)-linked whole antibody (1:10000) (Amersham Pharmacia Biotech, UK) or anti-mouse IgG HRP-linked whole antibody (ab97046, 1:20000) (Abcam, Cambridge, UK) were used. HRP-immunoreactive bands were developed by Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences, Chicago, IL, USA) and developed using X-ray films (Kodak, Rochester, NY, USA) or detected using iBright FL1500 Imaging System (Thermo Fisher Scientific, MA, USA). Image J analysis PC software (NIH Bethesda, MD, USA) or iBright FL1500 Imaging System Software were used to analyse protein bands densitometry. β-actin was used as equal loading control in all samples.

Statistical analysis

All data are presented as the means±S.E.M. A normal distribution of the obtained data was tested by the Shapiro-Wilk test. For the normally distributed data, main effects of the fructose diet and chronic stress, as well as their interaction, were evaluated using two-way ANOVA, while inter-group differences were determined by the post-hoc Tukey test. Statistical significance was accepted at $p<0.05$. All analyses were performed using STATISTICA 8.0 software (Stat Soft Inc., USA).

RESULTS

Food, liquid and energy intake

We analysed food, liquid and total energy intake to reveal the effects of stress and dietary fructose and their combination on physiological parameters related to eat-

Table 1. Food, liquid and energy intake of control rats, fructose-fed control rats, rats exposed to chronic unpredictable stress, and fructose-fed stressed rats.

	C	C+F	S	S+F
Food intake (kJ/day/cage)	57.2±1.9	36.7±1.3***	57.6±1.1	33.8±2.0***†††
Liquid intake (kJ/day/cage)	117.1±6.7	150.7±2.7***	103.2±1.7	151.4±2.6***†††
Energy intake (kJ/day/cage)	629.7±27.4	922.1±20.4***	633.5±12.4	892.6±30.1***†††

The data are presented as means ±S.E.M. (n=3 cages/3 animals per cage). A value of $p<0.05$ was considered statistically significant. Significant between-groups differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows: ***$p<0.001$, treated animals *vs.* C †††$p<0.001$, S+F *vs.* S

ing. Two-way ANOVA showed the effect of the liquid fructose-enriched diet on food intake ($F(1, 32)=147.29$, $p<0.001$), liquid intake ($F(1, 32)=85.25$, $p<0.001$) and total energy intake ($F(1, 32)=130.89$, $p<0.001$). As shown in Table 1, post-hoc test revealed that both experimental groups consuming 20% fructose solution had decreased average food intake during the 9-week period (***$p<0.001$, C+F *vs.* C; S+F *vs.* C). These animals drank more liquid fructose (***$p<0.001$, C+F *vs.* C; S+F *vs.* C) and hence, they consumed more total kJs than rats drinking water (***$p<0.001$, C+F *vs.* C; S+F *vs.* C). In addition, fructose-fed stressed rats also consumed less food but more total kJ in comparison to the stress alone (Table 1, ††† $p<0.001$, S+F *vs.* S).

Physiological and biochemical parameters

The body mass of rats on the fructose diet was not significantly different from the body mass of animals kept on standard diet, despite higher energy intake in fructose-fed rats, as revealed by two-way ANOVA (Fig. 1a). However, two-way ANOVA revealed significant effects of both fructose diet ($F(1, 20)=13.64$, $p<0.01$) and stress ($F(1, 20)=4.80$, $p<0.05$) on adiposity index, calculated as VAT mass to body mass ratio. As shown in Fig. 1b, post-hoc test showed reduced VAT-to-body mass ratio in stressed rats compared to the control animals (*$p<0.05$, S *vs.* C), while in fructose-fed stressed rats this ratio was significantly increased in comparison to the stressed rats on standard diet (†† $p<0.01$, S+F *vs.* S).

To assess the physiological effects of the applied stress protocol, plasma corticosterone level were measured. Two-way ANOVA detected significant effects of stress ($F(1, 28)=34.86$, $p<0.001$), fructose ($F(1, 28)=17.87$, $p<0.001$) and their interaction ($F(1, 28)=17.02$, $p<0.001$) on corticosterone level in plasma. As shown in Fig. 1c, post-hoc test showed that stressed animals on standard diet had significantly elevated plasma corticosterone concentrations (***$p<0.001$, S *vs.* C). However, fructose diet decreased plasma corticosterone level in stressed rats to the level comparable to the controls (††† $p<0.001$, S+F *vs.* S).

Significant effect of fructose diet on plasma insulin level was detected by Two-way ANOVA ($F(1, 20)=42.16$, $p<0.001$), while post-hoc test showed increased plasma insulin level in fructose-fed stressed rats in comparison to both control (Fig. 1d, *$p<0.05$, S+F *vs.* C) and stressed rats on standard diet (†† $p<0.01$, S+F *vs.* S).

Leptin signalling

Two-way ANOVA detected a significant effect of stress ($F(1, 24)=36.24$, $p<0.001$) on the plasma leptin level. As shown in Fig. 2a, post-hoc test revealed that plasma leptin levels were reduced in stressed rats compared to the control animals (**$p<0.01$, S *vs.* C), while trend of increase ($p=0.07$) was detected in stressed rats fed with fructose in comparison to the stressed rats on standard diet.

In the case of hypothalamic ObRb mRNA level, two-way ANOVA detected a significant interaction between dietary fructose and stress ($F(1, 24)=13.78$, $p<0.01$). As shown in Fig. 2b, post-hoc test revealed that stress led to reduced hypothalamic mRNA levels of ObRb (*$p<0.05$, S *vs.* C), while increased ObRb mRNA expression was detected in stressed rats on fructose diet in comparison to stressed animals on standard diet (Fig. 2b, †$p<0.05$, S+F *vs.* S). As for SOCS3 expression, two-way ANOVA revealed significant effects of both fructose feeding ($F(1, 24)=6.59$, $p<0.05$) and chronic stress ($F(1, 24)=14.16$, $p<0.001$), while post-hoc test showed that SOCS3 mRNA level was significantly decreased in the hypothalamus of stressed rats on standard diet in comparison to the control group of animals (Fig 2b, *$p<0.05$, S *vs.* C).

Glucose transporters and insulin signalling in the hypothalamus

As shown in Fig. 3a, fructose diet and/or stress did not affect hypothalamic GLUT1, GLUT2 and GLUT3

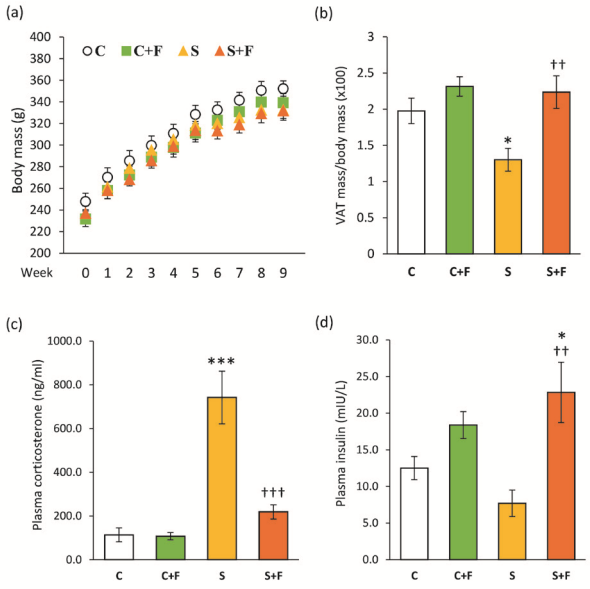

Figure 1. Physiological and biochemical parameters.
(a) Body mass (recorded weekly); **(b)** VAT mass to body mass ratio (at the end of the experiment); **(c)** plasma corticosterone level (ng/ml) and; **(d)** plasma insulin (mIU/L) level of C – control, C+F – fructose-fed control, S – stressed and S+F – fructose-fed stressed rats during 9 weeks on fructose diet combined with 4 weeks of chronic unpredictable stress. Data are presented as means ± S.E.M. (n=6–8 animals per group). To evaluate the effects of fructose and stress, and their interaction on treated groups, two-way ANOVA was used. A value of $p<0.05$ was considered statistically significant. Post-hoc Tukey test was used to analyse inter-group differences. Statistically significant differences were marked as follows: *$p<0.05$, ***$p<0.001$, treated animals *vs.* C; ††$p<0.01$, †††$p<0.001$, S+F *vs.* S.

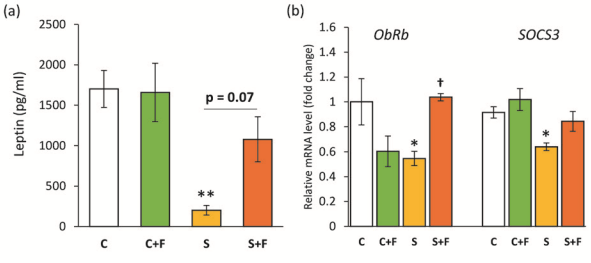

Figure 2. Leptin signalling.
(a) Plasma leptin levels and **(b)** mRNA levels of *ObRb* and *SOCS3* (normalized to *HPRT1* mRNA) of C – control, C+F – fructose-fed control, S – stressed and S+F – fructose-fed stressed rats during 9 weeks on fructose diet combined with 4 weeks of chronic unpredictable stress. Data are presented as means±SEM (n=8 animals per group). To evaluate the effects of fructose and stress, and their interaction on treated groups, two-way ANOVA was used. A value of $p<0.05$ was considered statistically significant. Post-hoc Tukey test was used to analyse inter-group differences. Statistically significant differences are marked as follows: *$p<0.05$, **$p<0.01$, treated animals *vs.* C; †$p<0.05$, S+F *vs.* S. ObRb, leptin receptor; SOCS-3, suppressor of cytokine signalling-3; HPRT1, hypoxanthine phosphoribosyl transferase 1.

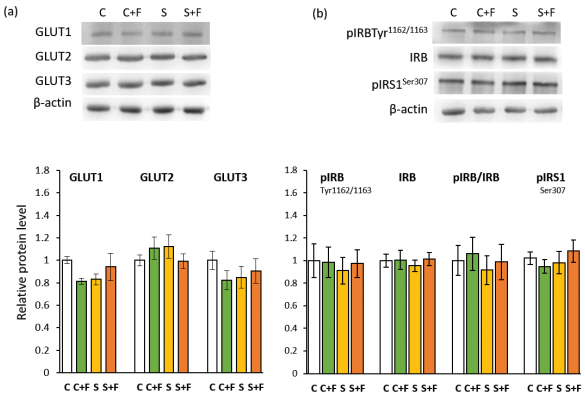

Figure 3. Glucose transporters and insulin receptor signalling in the hypothalamus.
Representative Western blots and relative quantification of **(a)** GLUT1, 2 and 3 transporters; **(b)** IR protein level and phosphorylation, IRS1 phosphorylation in hypothalamic protein extract of C – control, C+F – fructose-fed control, S – stressed and S+F – fructose-fed stressed rats during 9 weeks on fructose diet combined with 4 weeks of chronic unpredictable stress. β-actin was used for the normalization of immuno-positive bands of target proteins, which are presented as mean ±S.E.M. (n=8 animals per group). To evaluate the effects of fructose and stress, and their interaction on treated groups, two-way ANOVA was used. A value of $p<0.05$ was considered statistically significant. GLUT, glucose transporter; IR, insulin receptor; IRS, insulin receptor substrate.

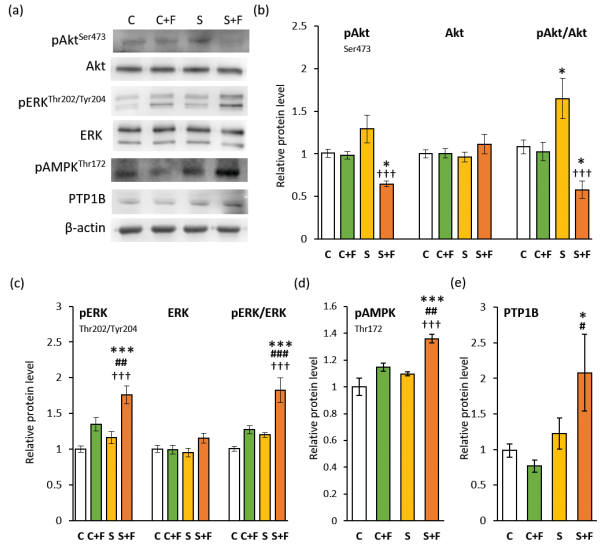

Figure 4. Akt, ERK, AMPK and PTP1B in the hypothalamus.
(a) Representative Western blots for pAkt Ser473, Akt, pERK Thr202/Tyr204, ERK, pAMPK Thr172 and PTP1B. Relative quantifications of **(b)** pAkt Ser473, Akt and their ratio; **(c)** pERK Thr202/Tyr204, ERK and their ratio **(d)** pAMPK Thr172 and **(e)** PTP1B and in the hypothalamus of C – control, C+F – fructose-fed control, S – stressed and S+F – fructose-fed stressed rats during 9 weeks on fructose diet combined with 4 weeks of chronic unpredictable stress. β-actin was used for the normalization of immuno-positive bands of target proteins, which are presented as means ±S.E.M. (n=8 animals per group). To evaluate the effects of fructose and stress, and their interaction on treated groups, two-way ANOVA was used. A value of $p<0.05$ was considered statistically significant. Post-hoc Tukey test was used to analyse inter-group differences. Statistically significant differences are marked as follows: $^{*}p<0.05$, $^{***}p<0.001$, treated animals *vs.* C; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, S+F *vs.* C+F; $^{\dagger\dagger\dagger}p<0.001$, S+F *vs.* S. Akt, protein kinase B; PTP1B, protein tyrosine phosphatase 1B.

protein content. Total IR, its phosphorylated form and their ratio (pIRTyr1162/1163/IR), as well as pIRS1Ser307 were also unaltered by fructose, stress and their combination in the whole cell extracts of the hypothalamus (Fig. 3b).

Although total protein level of Akt was not changed, in the case of pAkt Ser473, Two-way ANOVA showed significant effect of fructose diet ($F(1, 32)=13.83$, $p<0.001$), as well as significant interaction between fructose diet and stress ($F(1, 32)=11.99$, $p<0.01$). The ratio of phosphorylated to total Akt was also affected by the fructose diet ($F(1, 28)=18.61$, $p<0.001$) and by the interaction between factors ($F(1, 28)=15.04$, $p<0.001$). Post-hoc test revealed (as shown in Fig. 4b) that both pAkt and pAkt/Akt ratio, were lower in stressed rats on fructose diet in comparison to the control rats ($^{*}p<0.05$, S+F *vs.* C) and standard-fed stressed animals ($^{\dagger\dagger\dagger}p<0.001$, S+F *vs.* S). In addition, pAkt/Akt ratio was also significantly higher in stressed animals on standard diet when compared to the unstressed rats on standard diet ($^{*}p<0.05$, S *vs.* C).

According to the two-way ANOVA, significant interaction between fructose and stress was detected in the case of ERK phosphorylation ($F(1, 31)=4.05$, $p<0.05$), as well as the effects of fructose diet ($F(1, 31)=17.89$, $p<0.001$) and stress ($F(1, 31)=9.31$, $p<0.01$). This was also evident in the case of pERK/ERK ratio, where also interaction of factors was observed ($F(1, 31)=4.53$, $p<0.05$) and both fructose diet ($F(1, 31)=28.09$, $p<0.001$) and stress ($F(1, 31)=19.64$, $p<0.001$) had significant effects. As shown in Fig. 4c and revealed by the post-hoc test, fructose-fed stressed rats exhibited significantly increased ERK phosphorylation and pERK/ERK ratio in comparison to the control rats ($^{***}p<0.001$, S+F *vs.* C), fructose alone ($^{\#\#}p<0.01$; S+F *vs.* C+F, for pERK and $^{\#\#\#}p<0.001$; S+F *vs.* C+F, for pERK/ERK ratio) and stress alone ($^{\dagger\dagger\dagger}p<0.001$, S+F *vs.* S).

Two-way ANOVA detected significant effects of both fructose diet ($F(1, 20)=41.26$, $p<0.001$) and stress ($F(1, 20)=23.75$, $p<0.001$) on AMPK phosphorylation at Thr172. As shown in the Fig. 4d, the stressed rats on fructose diet had higher hypothalamic AMPK phosphorylation than the control rats on standard diet ($^{***}p<0.001$, S+F *vs.* C), fructose-fed control rats ($^{\#\#}p<0.01$; S+F *vs.* C+F) and stressed rats on standard diet alone ($^{\dagger\dagger\dagger}p<0.001$, S+F *vs.* S).

Finally, stress had significant effect on PTP1B protein level as shown by Two-way ANOVA ($F(1, 31)=7.79$, $p<0.01$). PTP1B protein level was also influenced by the interaction of fructose feeding and stress ($F(1, 31)=3.74$, $p<0.05$). As shown in Fig. 4e, post-hoc test revealed that PTP1B was elevated in fructose-fed stressed rats in comparison to both control on standard and on fructose diet ($^{*}p<0.05$, S+F *vs.* C; $^{\#}p<0.05$, S+F *vs.* C+F).

Appetite-regulating neuropeptides in the hypothalamus

Expression of orexigenic AgRP and anorexigenic POMC appetite-regulating neuropeptide was analysed. In the case of AgRP, two-way ANOVA revealed effect of fructose diet ($F(1, 27)=5.01$, $p<0.05$) and significant interaction between fructose diet and stress ($F(1, 27)=4.75$, $p<0.05$) on the mRNA level of this orexigenic neuropeptide. As shown in Fig. 5, post-hoc test revealed that the combination of fructose and stress significantly increased the mRNA level of AgRP neuropeptide in comparison to all other experimental groups ($^{*}p<0.05$, S+F *vs.* C; $^{\#}p<0.05$, S+F *vs.* C+F; $^{\dagger}p<0.05$, S+F *vs.* S).

Two-way ANOVA detected a significant effect of stress on POMC ($F(1, 24)=30.47$, $p<0.001$) mRNA level. According to the post-hoc analysis, expression of

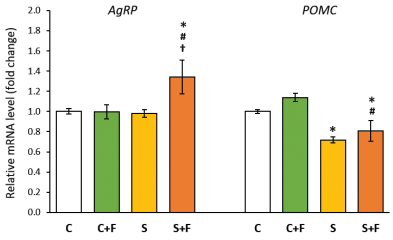

Figure 5. Expression of appetite-regulating neuropeptides.
The mRNA levels of *AgRP* and *POMC* (normalized to *HPRT1* mRNA) were determined in the hypothalamus of C – control, C+F – fructose-fed control, S – stressed and S+F – fructose-fed stressed rats during 9 weeks on fructose diet combined with 4 weeks of chronic unpredictable stress. Data are presented as means ± S.E.M. (n=8 animals per group). To evaluate the effects of fructose and stress, and their interaction on treated groups, two-way ANOVA was used. A value of $p<0.05$ was considered statistically significant. Post-hoc Tukey test was used to analyse inter-group differences. Statistically significant differences are marked as follows: *$p<0.05$, treated animals *vs.* C; #$p<0.05$, S+F *vs.* C+F; †$p<0.05$, S+F *vs.* S. POMC, proopiomelanocortin; AgRP, agouti-related protein; HPRT1, hypoxanthine phosphoribosyl transferase 1.

anorexigenic POMC neuropeptide in the hypothalamus was decreased in stressed rats on standard diet (Fig 5, *$p<0.05$, S *vs.* C) and in fructose-fed stressed rats in comparison to both control rats on standard and fructose diet (Fig 6, *$p<0.05$, S+F *vs.* C; #$p<0.05$, S+F *vs.* C+F).

DISCUSSION

Food intake regulation is based on a complex network of central and peripheral signals integrated in the hypothalamus, a brain structure which responds with coordinated release of orexigenic and anorexigenic neuropeptides that finally inhibit or promote appetite. Disturbances in the appetite regulation in the hypothalamus could arise from the excessive consumption of sugar-rich food, but everyday exposure to stress should not be neglected as a contributing factor. Namely, under stress conditions, appetite signals do not work adequately resulting in the disturbed expression of the appetite-related neuropeptides. And while certain individuals respond to stress conditions with decreased appetite, others consume food more readily. Thus, our goal was to investigate the combined effects of liquid fructose intake and chronic unpredictable stress, through leptin and insulin signalling, on the regulation of appetite control in the hypothalamus of male rats. Our results showed that only combination of dietary fructose and stress led to concomitant increase of orexigenic *AgRP* and decrease of anorexigenic *POMC* expression. This state was associated with higher activity of AMPK energy sensor despite increased plasma insulin level, as well as with signs of insulin resistance reflected in reduced Akt and enhanced ERK activity and elevated PTP1B protein level in the hypothalamus.

Excessive fructose ingestion has been previously related with problems in energy intake regulation (Bray *et al.*, 2004). Indeed, in the current study, all rats on the diet enriched with liquid fructose exhibited elevated energy intake, which was most likely a consequence of fructose-ingested calories, since solid food intake was reduced in these animals. However, this higher energy intake was translated neither in the increased body mass of these animals, nor in the higher VAT/body ratio. Unchanged body mass has also been previously evidenced in other studies with a fructose diet, including ours (Bursac *et al.*, 2013; Bursac *et al.*, 2014; Shapiro *et al.*, 2008). It is noteworthy that some studies using fructose diet reported an increased VAT mass. The majority of these studies, including the ones from our laboratory, were conducted on females (Kovacevic *et al.*, 2014; Sangüesa *et al.*, 2017; Yoo *et al.*, 2017; Kovacevic *et al.*, 2017; Nyakudya *et al.*, 2018; Brkljačić *et al.*, 2019), which could point that the fructose effects on VAT accumulation might be sex-related. Furthermore, discrepancies between previous studies on males and our current findings might originate from differences in the strain of the experimental animals, since more prominent effects of fructose diet on adiposity were observed in Sprague-Dawley rats (Bocarsly *et al.*, 2010; Crescenzo *et al.*, 2014; Nyakudya *et al.*, 2018; Zubiría *et al.*, 2013). Finally, as recently reviewed by Chan and others (Chan *et al.*, 2021), the concentration of administered fructose solution (ranging from 10% to 60%) (Bursac *et al.*, 2014; de Moura *et al.*, 2009), as well as the treatment duration (Dupas *et al.*, 2018; Chan *et al.*, 2021) and the age of the animals at the beginning of the experiment (suckling, young or adults) (Nyakudya *et al.*, 2018; Ramos *et al.*, 2017) could also be significant factors for the outcome of fructose diet on VAT mass.

Body mass also remained unchanged in stressed rats on the standard diet, however the VAT/body mass ratio was notably decreased. This is most likely a consequence of very high corticosterone plasma concentrations observed in response to stress in these animals, that may have stimulated fat loss through glucocorticoid-induced lipolysis in the adipose tissue (Bursac *et al.*, 2018). The observed decrease of the VAT/body mass ratio was paralleled with a decrease of circulatory leptin concentration, which goes in line with findings of Weigle and others (Weigle *et al.*, 2003). In addition, expression of hypothalamic *ObRb* and *SOCS3* was downregulated after stress, which is also in accordance with decreased circulatory leptin level. On the other hand, in fructose-fed stressed rats the VAT/body mass ratio was higher than in standard-fed stressed animals, most likely due to the inhibition of adipose tissue lipolysis previously shown after the high-fructose diet (Abdel-Sayed *et al.*, 2008). The observed change of visceral adiposity in fructose-fed stressed rats was accompanied with the same change in *ObRb* expression and a trend of leptin increase ($p=0.07$). Such anti-stress effect of fructose could be the result of both inherent ability of fructose to induce leptin increase (Shapiro *et al.*, 2008, 2011) and normalization of stress-induced corticosterone by fructose, since glucocorticoids play a key inhibitory role in the action of leptin (Zakrzewska *et al.*, 1997). The blunting of stress-induced plasma corticosterone level by fructose diet is in accordance with previous study analysing high-sucrose diet combined with chronic restraint stress (Corona-Pérez *et al.*, 2017). This anti-stress effect of fructose is most likely a consequence of the diminished central reactivity to stress of rats on hypercaloric diet (De Oliveira *et al.*, 2015; MacEdo *et al.*, 2012) or could be a result of reward-based eating meant to reduce stress response (Adam & Epel, 2007). Alternatively, the mechanism behind neuroprotective effect of fructose, which leads to restoration of stress-disturbed leptin signalling to the level observed in the control animals, could lay in the ability of fructose and its metabolites to prevent changes of oxidative status induced by chronic unpredictable stress (Spasojević *et al.*, 2009; Che *et al.*, 2015). It is noteworthy that fructose can play a divergent role in the living cells, both protective and detrimental, depending on the concentration and duration of the fructose treatment, as well as on the conditions in the cell (Semchyshyn, 2013).

Although fructose metabolism has been considered to be independent of insulin, a study of Lindqvist and others (Lindqvist *et al.*, 2008) showed that serum insulin levels could be significantly affected by drinking of fructose. In the present study, the fructose diet alone did not have significant impact on insulin level, but when combined with stress, significantly higher plasma insulin concentrations were observed. Insulin is considered as an important peripheral signal involved in the hypothalamic appetite-regulation and the local role of insulin signalling in this brain region has been recognized as essential in the energy balance regulation and maintenance of glucose homeostasis (Vogt & Bruning, 2013). However, the results of the present study showed unchanged GLUT1, GLUT2 and GLUT3 protein levels in the hypothalamus suggesting that efficient glucose uptake at the level of insulin-regulated glucose transporters was not disturbed (Shah *et al.*, 2012). This was also true for the IR protein level and activity, as well as for the inhibitory phosphorylation of IRS1 on serine 307. Although both IR and IRS1 activities were unchanged, activities of other mediators of insulin signalling, ERK and Akt, were altered in stressed rats on fructose-enriched diet. Namely, ERK was hyperphosphorylated at Thr^{202}/Tyr^{204}, while Akt was dephosphorylated at Ser^{473} in the hypothalamus of these animals. The results of enhanced ERK diminished Akt activation have been previously implicated in the state of insulin resistance (Jiang *et al.*, 2003; Ozaki *et al.*, 2016). Our previous findings in female rats subjected to fructose feeding and chronic unpredictable stress also showed diminished hypothalamic insulin signalling at the level of Akt (Kovacevic *et al.*, 2019), while other studies reported decreased pAkt-Ser^{473} and unaltered pIRS1-Ser^{307} after 4 months of sugar diet (Battu *et al.*, 2012), and decreased phosphorylation of Akt after 4 weeks of high-fructose diet (Zhang *et al.*, 2014).

It has been previously shown, in differentiated hippocampal neurons and different cell lines, that Akt dephosphorylation occurs in response to AMPK activation (King *et al.*, 2006; Kim *et al.*, 2009). Indeed, our results confirmed an increased level of AMPK activatory phosphorylation in the hypothalamus of fructose-fed stressed rats, which was concomitant with Akt serine 473 dephosphorylation. It is noteworthy that circulatory insulin is a known inhibitory signal of the hypothalamic AMPK (Huynh *et al.*, 2016), but nonetheless, this anorexigenic effect of insulin was absent herein, judged by significantly decreased *POMC* mRNA level paralleled with prominent increase in the expression of orexigenic *AgRP*. This was the case only when stress was combined with the fructose-enriched diet. Since AMPK is recognized as an important regulator of appetite, which (in response to fasting) positively affects expression of orexigenic and decreases the level of anorexigenic neuropeptides (Kola, 2008; Huynh *et al.*, 2016) it could be proposed that the observed changes in neuropeptides in the direction of appetite promotion could be a direct consequence of increased AMPK activity (Claret *et al.*, 2007; Oh *et al.*, 2016). Possible explanation of increased AMPK activity, despite higher circulatory insulin and increased energy intake (Minokoshi *et al.*, 2004), comes from the studies showing that stress hormones i.e. glucocorticoids, could exert a direct stimulatory effect on AMPK activity in rat hypothalamus (Christ-Crain *et al.*, 2008). It is interesting to speculate that this stress-related effects on AMPK activity could then rather be ascribed to the endocannabinoid system (Hill & Tasker, 2012). Namely, the cannabinoid receptor type 1 was revealed as the necessary mediator for the effects of stress on hypothalamic AMPK activity (Scerif *et al.*, 2013) and the orexigenic effects of cannabinoids have been previously related to stimulated AMPK activity (Kola *et al.*, 2005; Thuijl *et al.*, 2007).

Finally, in the hypothalamus of fructose-fed stressed rats, we also observed significant increase of PTP1B, a major tyrosine phosphatase implicated in both leptin and insulin resistance and upregulated in chronic overeating (Ono, 2019; Morrison *et al.*, 2007; Dodd *et al.*, 2019). A direct effect of PTP1B deficiency has been shown to result in the attenuation of AgRP mRNA expression under the high calorie diet (Sugiyama *et al.*, 2017). It is noteworthy that PTP1B regulated adiposity and leptin sensitivity likely involves coordinated regulation of AMPK in hypothalamus and peripheral tissues (Xue *et al.*, 2009).

Taken together, our results show that combination of diet enriched with liquid fructose and chronic stress promotes appetite through concomitant increase of *AgRP* and decrease of *POMC* expression, activates AMPK energy sensor, disturbs Akt and ERK signalling and elevates PTP1B protein level. These findings are pointing that the joint effects of dietary fructose and chronic stress are more deleterious than the separate ones, since the inappropriate appetite control may reflect a sustained drive to consume food and provide a setting for the disturbed energy homeostasis in the long run.

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