



Impairment of Novel Object Recognition Memory and Brain Insulin Signaling in Fructose- but Not Glucose-Drinking Female Rats

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Received: 17 July 2017 / Accepted: 22 December 2017 / Published online: 26 January 2018
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Abstract

Excessive sugar intake has been related to cognitive alterations, but it remains unclear whether these effects are related exclusively to increased energy intake, and the molecular mechanisms involved are not fully understood. We supplemented Sprague-Dawley female rats with 10% *w/v* fructose in drinking water or with isocaloric glucose solution for 7 months. Cognitive function was assessed through the Morris water maze (MWM) and the novel object recognition (NOR) tests. Plasma parameters and protein/mRNA expression in the frontal cortex and hippocampus were determined. Results showed that only fructose-supplemented rats displayed postprandial and fasting hypertriglyceridemia (1.4 and 1.9-fold, $p < 0.05$) and a significant reduction in the discrimination index in the NOR test, whereas the results of the MWM test showed no differences between groups. Fructose-drinking rats displayed an abnormal glucose tolerance test and impaired insulin signaling in the frontal cortex, as revealed by significant reductions in insulin receptor substrate-2 protein levels (0.77-fold, $p < 0.05$) and Akt phosphorylation (0.72-fold, $p < 0.05$), and increased insulin-degrading enzyme levels (1.86-fold, $p < 0.001$). Fructose supplementation reduced the expression of antioxidant enzymes and altered the amount of proteins involved in mitochondrial fusion/fission in the frontal cortex. In conclusion, cognitive deficits induced by chronic liquid fructose consumption are not exclusively related to increased caloric intake and are correlated with hypertriglyceridemia, impaired insulin signaling, increased oxidative stress and altered mitochondrial dynamics, especially in the frontal cortex.

Keywords Simple sugars · Cognitive deficit · Frontal cortex · Hippocampus · Metabolic dysfunctions

Juan Carlos Laguna and Marta Alegret contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12035-017-0863-1>) contains supplementary material, which is available to authorized users.

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Introduction

Metabolic diseases such as obesity, type 2 diabetes (T2D), and non-alcoholic steatohepatitis have been linked to cognitive disorders [1]. Specifically, cognitive decrements, i.e., slight changes in cognitive performance that do not interfere with daily life, have been observed not only in T2D patients, but also in individuals with pre-diabetic insulin resistance (IR) [2]. However, both metabolic and cognitive alterations are complex, multifactorial diseases, and epidemiological studies do not demonstrate a causal relationship between them or the mechanisms involved.

It is widely acknowledged that a high intake of carbohydrates, particularly simple sugars, is associated with metabolic pathologies (obesity, IR and T2D) [3–6]. More recently, sugar intake has also been related to cognitive alterations [7–9]. The intake of simple sugars in the human diet has risen in the last decades and it is particularly high in industrialized countries, especially due to the elevated consumption of sugar-sweetened beverages (SSB) [10]. These beverages are usually sweetened

with sucrose or with high-fructose corn syrup (HFCS), containing roughly 50% glucose and 50% fructose. Although glucose and fructose molecules are very similar, fructose seems to be more harmful than glucose to human health, due to its particular metabolic effects [11, 12]. In this sense, our research group has demonstrated that liquid fructose supplementation in rats for a 2-month period causes worse metabolic and vascular responses than liquid glucose, despite the similar or even higher caloric intake of glucose-supplemented rats [13, 14]. However, to our knowledge there are no reports comparing the effects of glucose and fructose on brain health. Moreover, it remains unclear whether these effects are related exclusively to the increased energy intake in individuals who consume large amounts of simple sugars. A population-based case-control study showed that a high caloric intake was associated with increased odds of developing mild cognitive impairment [15]. Nevertheless, the type of nutrient that provides these calories may also be a determining factor: a population-based prospective study performed with elderly subjects showed a higher risk of mild cognitive impairment or dementia in individuals who obtained a higher percentage of their daily calories from carbohydrates than from fat and proteins [16].

Female animals are underrepresented in preclinical studies, and there is increasing awareness by the National Institutes of Health (NIH) that research should include females. In our previous studies, we showed that female rats supplemented with liquid fructose displayed a more detrimental response than male rats, including glucose intolerance and insulin resistance, which were not observed in males [17]. Based on these results, and taking into account the putative relationship between metabolic abnormalities and cognitive alterations, in this study, we used female Sprague–Dawley rats.

In our previous studies, we used a 2-month fructose or glucose supplementation period, which is roughly equivalent to six human years of daily sugar consumption [18]. However, the pattern of sugar-sweetened beverage consumption in humans may extend for much longer periods; in addition, both metabolic and cognitive alterations evolve over many years. Thus, in the present work, we attempted to examine whether long-term (7 months) supplementation with liquid glucose or fructose, providing the same amount of calories, causes not only metabolic but also cognitive alterations in female rats, and to explore the molecular mechanisms involved.

Materials and Methods

Animals and Diets

Female Sprague–Dawley rats were obtained from Charles River (Barcelona, Spain), and were maintained under conditions of constant humidity (40–60%) and temperature (20–24 °C) with a normal light/dark cycle of 12 h. Procedures were

conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee (Autonomous Government of Catalonia Act 5/1995, of July 21). All experimental procedures involving animals were approved by the University of Barcelona's Animal Experimentation Ethics Committee (approval no. 7912).

Thirty-six adult rats (aged 8 weeks) were randomly assigned to either a control group (no supplementary sugar, $n = 12$), a fructose-supplemented group (10% *w/v* in drinking water) ($n = 12$) or a group supplemented with a glucose solution ($n = 12$), for 7 months (28 weeks). The concentration of the glucose solution was calculated in order to match the amount of calories ingested by the fructose group, as in our previous report [13]. All the animals received 2014 Teklad rodent maintenance diet (Envigo, Barcelona, Spain). Throughout the treatment, the amount of solid food ingestion was controlled weekly and liquid consumption three times a week, by weighing the food pellets or measuring the volume of liquid provided, and subtracting the amount left on the day of control. Body weight was assessed every 2 weeks.

Glucose Tolerance Test

A glucose tolerance test (GTT) was performed at week 26 after the beginning of the sugar supplementation period, as described previously [13]. Briefly, after a 6 h fast (8 a.m.–2 p.m.), and following the collection of an unchallenged sample (time 0), a glucose solution of 2 g/kg body weight was administered into the peritoneal cavity. During the test, blood was collected from the tail vein at 15, 30, 60, 90, and 120 min after glucose administration. Glucose measurements were performed using a hand-held glucometer (Accutrend® Plus System Cobas, Roche Farma, Barcelona, Spain). Plasma insulin levels were measured at baseline, 15, 60, and 120 min post glucose administration using a rat insulin ELISA kit (Millipore, Billerica, MA, USA).

Cognitive Tests

Cognitive tests (novel object recognition (NOR) and Morris water maze (MWM) tests) were performed twice during the study, on week 2 and week 27 after the beginning of the sugar supplementation period.

The NOR task procedure consisted of habituation, familiarization, and test phases. In the habituation phase, each animal was placed in an empty test arena (30 × 70 × 70 cm) and allowed to explore it for 10 min for 2 days. On the third day, each rat was placed in the same open-field arena, which contained two identical objects (A + A) placed side by side, for 10 min (familiarization phase). Following the familiarization phase, after a delay of 2 h (first test, short-term memory) and 24 h (second test, long-term memory), the animal was returned to the arena with two objects, one of which was

identical to the object used in the familiarization phase and the other was a novel object, for 10 min. The novel object was different in the first and second test (A+B and A+C, respectively). The objects used in this experiment were mostly small toys (8–12 cm) with a variety of textures, shapes, colors, and sizes, which were fixed on the floor with removable adhesive tape with their edges at 15 cm from the walls. The precise position of each object was always the same, the left/right position of the novel object was counterbalanced with each group, and new pairs of objects were used for each NOR test. The behavior of the rats during the tests was recorded with a camera. For each animal, we measured the total exploration time during the familiarization phase in order to confirm the correct memory evaluation, and the time spent exploring the novel object (TN) and the old object (TO) during the test phase. A discrimination index (DI) was defined as $(TN - TO)/(TN + TO)$. The arena and objects were cleaned with 96% ethanol after each test in order to eliminate olfactory cues.

The MWM tests were performed 2 days after finishing the NOR tests, using an open circular pool (160 cm in diameter, 50 cm in height) half filled with water at a temperature of $22\text{ }^{\circ}\text{C} \pm 1$. Two principal perpendicular axes were defined; thus, the water surface was divided into four quadrants (NE, SE, SW, and NW), and five starting points were set (NE, E, SE, S, and SW). Four visual cues were placed on the walls of the tank (N, E, S, and W). Non-toxic white latex paint was added to make the water opaque, and a white escape platform (11 cm in diameter) was submerged 1 cm below the water level (approximately in the middle of one of the quadrants). The position of the platform was different in the tests performed after 2 weeks and after 7 months of sugar supplementation. The learning phase consisted of 6 days of trials for each rat. The animals were submitted to four trials each day and started from randomly set positions. There were no resting phases between trials. In each trial, the rat was gently placed in the water, facing the wall of the pool, and allowed to swim for 90 s. If the rat was unable to locate the platform during this period, it was guided or placed on the platform by the researcher. Animals were left on the platform for 30 s to allow them to spatially orient themselves. The animals' swimming paths were recorded by a video camera mounted above the center of the pool and the data were analyzed with SMART software version 3.0. The parameters measured were latency to find the platform, time spent in each quadrant and distance swam for each trial. A memory test was performed on day 7 whereby the platform was removed and the time spent by each rat in each quadrant measured.

Sample Preparation

Three days after finishing the cognitive tests, the rats were fasted 12 h. Eight rats from each group were anesthetized with

ketamine/xylazine (9 mg and 40 $\mu\text{g}/100\text{ g}$ body weight, respectively) and blood was collected by cardiac puncture. Plasma was prepared by centrifugation at $3000\times g$ for 10 min at room temperature. The rats were then euthanized by exsanguination, their brains dissected, and the frontal cortex and the hippocampus separated, immediately frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until needed.

The remaining four rats in each group were used for immunohistochemical studies. To this end, the rats were intracardially perfused with 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were then extracted and fixed in the same perfusion solution for 24 h at $4\text{ }^{\circ}\text{C}$. The samples were maintained for 24 h in 4% paraformaldehyde/30% sucrose solution, then frozen and stored at $-80\text{ }^{\circ}\text{C}$.

Blood and Plasma Parameters

Triglycerides (postprandial and after a 12 h fast) and fasting glucose levels were measured in blood samples using an Accutrend® Plus system glucometer (Cobas, Roche Farma, Barcelona, Spain). Concentrations of insulin and advanced glycation end products (AGE) in plasma were determined using specific enzyme-linked immunosorbent assay kits (Millipore, Billerica, MA, USA and MyBioSource, San Diego, CA, USA, respectively). Thiobarbituric acid reactive substance (TBARS) levels in plasma were determined using a colorimetric assay kit from Cayman (Ann Arbor, MI, USA).

RNA Preparation and Analysis

Total RNA was isolated with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. RNA concentration and purity were measured spectrophotometrically using the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). The ratios of absorbance at 260/230 and 260/280 were used as indicators of RNA purity. Specific mRNAs were assessed by real-time reverse transcription polymerase chain reaction (RT-PCR) using SYBR Green PCR Master Mix, specific primers and the Applied Biosystems StepOnePlus Sequence Detection System (Applied Biosystems, Foster City, CA, USA). β -actin (*actb*) was used as an internal control. Primer sequences and PCR product length are listed in Supplementary Table 1.

Preparation of Protein Extracts

Total protein extracts from the frontal cortex and hippocampus were obtained with the Helenius method [19]. Briefly, RIPA Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA) with protease, phosphatase and acetylase inhibitors (10 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 mM Nappi, 1 mM PMSF, 2 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ aprotinin, 1 mM Na_3VO_4 , 10 mM NaM, 1 μM TSA) was added to micronized tissue

and homogenized for 1.5 h at 4 °C. The samples were then centrifuged at 15,000×g for 15 min at 4 °C and the supernatant collected. Protein concentrations were determined using the Bradford method [20].

Western Blot Analysis

Different protein extracts (20–30 µg) from rat frontal cortex and hippocampus were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA), blocked for 1 h at room temperature with 5% non-fat milk solution in Tris-buffered saline (TBS) with 0.1% Tween-20, and incubated as described previously [21]. Detection was performed using the ECL chemiluminescence kit for HRP (Amersham GE Healthcare Europe GmbH, Barcelona, Spain). To confirm the uniformity of protein loading, blots were incubated with β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA), which was used as the normalization control for Western blot analysis. Primary antibodies for total and phosphorylated Akt, and total and phosphorylated GSK3β were supplied by cell signaling (Danvers, MA, USA), and those for Drp1, IDE, Mfn2, and PSD95 were obtained from Abcam (Cambridge, UK). The anti-SYP antibody was obtained from Dako (Agilent Technologies, Santa Clara, CA, USA), Opa1 from BD Biosciences (San Jose, CA, USA), total Tau from Chemicon International (Temecula, CA, USA), and phosphorylated Tau from Invitrogen (Carlsbad, CA, USA).

Brain-Derived Neurotrophic Factor

Levels of brain-derived neurotrophic factor (BDNF) protein were determined by an ELISA kit from Millipore (Billerica, MA, USA) using protein extracts from the frontal cortex and hippocampus.

Glial Fibrillary Acidic Protein Detection by Immunohistochemistry

Frozen brains were embedded in OCT cryostat embedding compound (Tissue-Tek, Torrance, CA, USA), cut into 25-µm-thick coronal sections using a cryostat (Leica Microsystems, Barcelona, Spain) at –23 °C, and placed on slides. The slides were stored at –20 °C in 0.1 M phosphate buffer containing 30% glycerol and 30% ethylene glycol. For the staining procedure, the brain sections were incubated for 2 h at room temperature with a blocking solution (0.1 M phosphate buffer containing 10% fetal bovine serum, 1% Triton, and 0.2% gelatin). The slices were then incubated overnight at 4 °C with an anti-glial fibrillary acidic protein (GFAP) antibody (1:1000, Dako, Agilent Technologies, Santa Clara, CA, USA). Next, the samples were incubated with a fluorescent

secondary antibody (Anti-Rabbit IgG Alexa Fluor 594, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) diluted in blocking solution for 2 h at room temperature. Nuclear staining was performed with 2 µg/mL of Hoechst H33342 (Sigma-Aldrich, Saint Louis, MO, USA) for 8 min at room temperature, and the sections were then mounted with Fluoromount-G™ (EMS, Hatfield, NJ, USA). Slides were allowed to dry overnight and images were acquired with a fluorescence laser microscope (Olympus BX41; Olympus, Hamburg, Germany). At least four images from four different animals per group were analyzed using ImageJ/1.49v software, available online from the National Institutes of Health, USA.

Statistical Analysis

The results are expressed as the mean of *n* values ± standard deviation. Plasma samples were assayed in duplicate. Gaussian distribution of the data was verified using the Kolmogorov–Smirnov normality test, and significant differences were established by one-way ANOVA and Šidák's post-hoc test for selected comparisons (GraphPad Software V6). When variance was not homogeneous, a non-parametric test (Kruskal Wallis) was performed. In addition, partial correlation after controlling for group was calculated using SPSS+21.00, between the variables of interest (see figure legend for details). Spearman's partial correlation coefficients between each possible pair of behavioral markers, plasma levels or neuronal markers were calculated with adjusted *p* values to eliminate false positive correlations. The statistical significance level was set at $p \leq 0.05$.

Results

Only Fructose Supplementation Significantly Increases Body Weight and Plasma Triglyceride, AGE, and TBARS Levels, Despite the Identical Total Calorie Consumption of the Glucose Group

Rats supplemented with liquid glucose or fructose for 7 months drank more than control rats (3.2- and 2.7-fold, respectively) and reduced their ingestion of solid food (0.6- and 0.7-fold, respectively) (Table 1). This reduction was not sufficient to compensate for the calories obtained from the sugars, and the total caloric intake was therefore increased by similar levels in both glucose and fructose groups (1.6 and 1.5-fold, respectively). However, only fructose-supplemented rats showed a significant increase in body weight throughout and at the end of the treatment period (Table 1). Moreover, only fructose-supplemented rats displayed postprandial and fasting hypertriglyceridemia (1.4

Table 1 Zoometric parameters and plasma analytes of female rats supplemented or not with 10% w/v liquid fructose or glucose for 7 months

	Control	Glucose	Fructose
AUC liquid intake (mL/[rat × 7 months]) ^a	7171 ± 923	22,935 ± 3340***	19,712 ± 5040**
AUC solid food intake (g/[rat × 7 months]) ^a	3190 ± 154	1899 ± 225***	2139 ± 310***
AUC total ingested kcal (kcal/[rat × 7 months]) ^a	9331 ± 443	14,705 ± 820***	14,119 ± 1430***
Final body weight (g) ^a	327 ± 23	344 ± 17	359 ± 38*
AUC weight (g/[rat × 7 months]) ^a	8103 ± 445	8267 ± 362	8623 ± 566*
Postprandial plasma triglycerides (mg/dL)	144 ± 24	147 ± 16	209 ± 45***###
Fasting plasma triglycerides (mg/dL)	155 ± 27	196 ± 50	292 ± 28***###
Plasma AGE (ng/mL)	12.2 ± 4.2	9.8 ± 6.7	22.9 ± 6.9*
Plasma TBARS (μmol/L)	23.2 ± 5.0	25.3 ± 4.3	34.1 ± 7.3*#

AUC area under the curve

Values are expressed as mean ± SD of $n = 8$ rats/group except for values labeled with ^a, which were obtained from $n = 12$ rats

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs glucose

and 1.9-fold) and increased levels of AGE (1.9-fold) and TBARS (1.5-fold).

Fructose but Not Glucose Supplementation Reduces Whole-Body Insulin Sensitivity and Alters the Glucose Tolerance Test

As shown in Fig. 1a, b, despite the fact that blood glucose levels were not altered, fructose-supplemented rats showed significant hyperinsulinemia at the end of treatment. The insulin sensitivity index therefore decreased significantly in this group only (Fig. 1c). The results of the GTT showed an increase in blood glucose levels 120 min after the glucose challenge in the fructose group only (Fig. 1d), but the integrated glucose concentration, calculated as the area under the curve (AUC), was not significantly altered (Fig. 1e). However, glucose-stimulated AUC insulin levels increased significantly in fructose-supplemented rats only (Fig. 1f, g).

Fructose Supplementation for 7 Months Impairs Novel Object Recognition

Rats were assessed in the NOR and MWM tests at the beginning (week 2) and at the end (week 27) of the sugar supplementation in order to study the short- and long-term effects on memory, respectively. The exploration times during the familiarization phase of the NOR procedure (Fig. 2a, d) were unaffected by sugar supplementation, and were long enough to ensure that the animals would

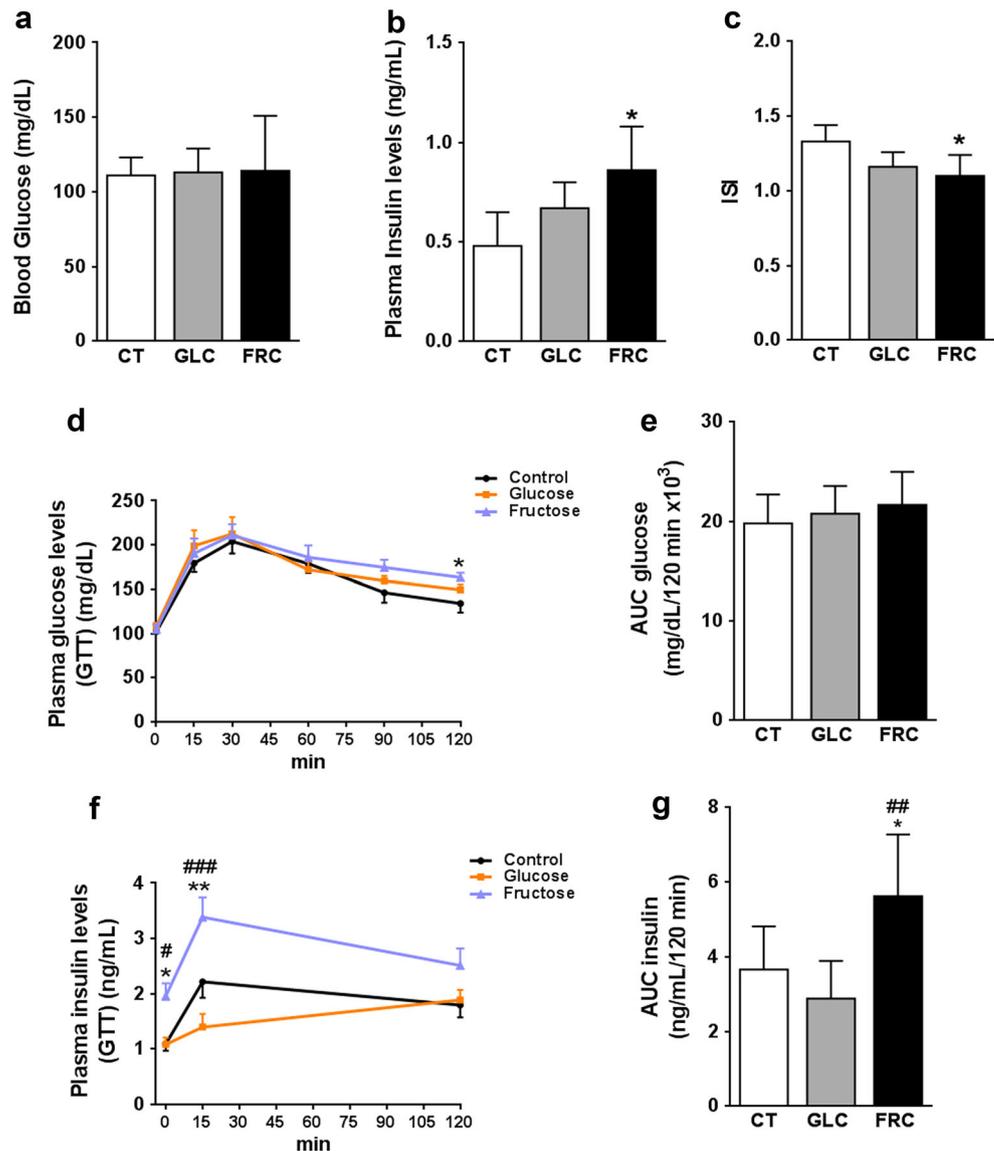
be able to distinguish the new object from the old one during the test. The results of the tests performed at week 2 did not reveal any effect of sugar supplementation on memory, since no differences were detected in the discrimination index (DI) between groups (Fig. 2b, c). By contrast, the NOR tests performed at week 27 showed a significant reduction in the discrimination index in the fructose group only (Fig. 2e, f).

The results of the MWM test showed no differences in spatial-learning acquisition and retention between the different experimental groups at the two time-points examined. Thus, all groups were able to learn over the course of the trial period (Fig. 3a, e) and the swimming velocity was similar (Fig. 3b, f). The removal test showed no differences in the percentage of time spent in the platform quadrant (Fig. 3c, g) or in the number of entries into this zone (Fig. 3d, h), defined as a quadrant area of 45 cm² around the platform.

Effects of Sugar Supplementation on Synaptic Plasticity-Related Parameters

Next, we determined the expression of several molecules involved in the regulation of synaptic plasticity, a mechanism underlying the acquisition and consolidation of learning and memory. The protein expression of brain-derived neurotrophic factor (BDNF) was reduced significantly by fructose supplementation in the hippocampus and also showed a trend towards a reduction in the frontal cortex of this group (Fig. 4a). However, the expression of other molecules also

Fig. 1 Blood glucose (a), plasma insulin levels (b), and insulin sensitivity index (c) from control (CT), glucose- (GLC), and fructose-supplemented rats (FRC). Results (mean \pm SD) were obtained the day that the animals were euthanized ($n = 8$ /group). Insulin sensitivity index is calculated from the formula $2/[(INS \times GLU) + 1]$, where INS is fasting plasma insulin levels (nM) and GLU is fasting blood glucose levels (μ M). **d–g** Results of the glucose tolerance test: plasma glucose (d) and insulin (f) values at different times after the intraperitoneal administration of a glucose solution (2 g/kg body weight). Area under the curve (AUC) values for glucose (e) and insulin (g) plasma concentrations represented in d and f. Results are the mean \pm SD of values from 11 to 12 animals/group. * $p < 0.05$, ** $p < 0.01$ vs CT; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs GLC



related to synaptic plasticity, such as synapsin1 (SYN1), growth-associated protein 43 (GAP-43), synaptophysin (SYP), and PSD95, was not altered by sugar supplementation (Fig. 4b–d).

Fructose, but Not Glucose, Alters Brain Insulin Signaling

It has been suggested that insulin signaling in the brain is involved in cognitive functions such as learning and memory [22]. Thus, the reduction in whole-body insulin sensitivity and the altered results of the NOR test in fructose-drinking rats prompted us to examine insulin signaling in the frontal cortex and the hippocampus. The mRNA expression of the main insulin-signaling transducers, insulin receptor substrates (IRS)-1 and -2, was not significantly modified (Fig. 5a). However, we found that IRS-2 protein

expression in the frontal cortex was significantly reduced in fructose-supplemented rats (Fig. 5b). Moreover, phosphorylation of V-akt murine thymoma viral oncogene homolog-2 (Akt) was also significantly reduced in the frontal cortex of the fructose group (Fig. 5c). A trend towards reduced Akt phosphorylation in the hippocampus was also observed, but did not reach statistical significance (Fig. 5d). The phosphorylation status of Akt-downstream molecules glycogen synthase kinase (GSK)3 β and Tau was not affected by sugar supplementation in either the frontal cortex or the hippocampus (Fig. 5c, d).

The expression of insulin-degrading enzyme (IDE), a protease involved in the degradation of both insulin and amyloid β (A β), was also determined. Rats supplemented with fructose showed a significant increase (1.8-fold, $p < 0.001$) in the IDE protein levels in the frontal cortex, but not in the hippocampus (Fig. 5e).

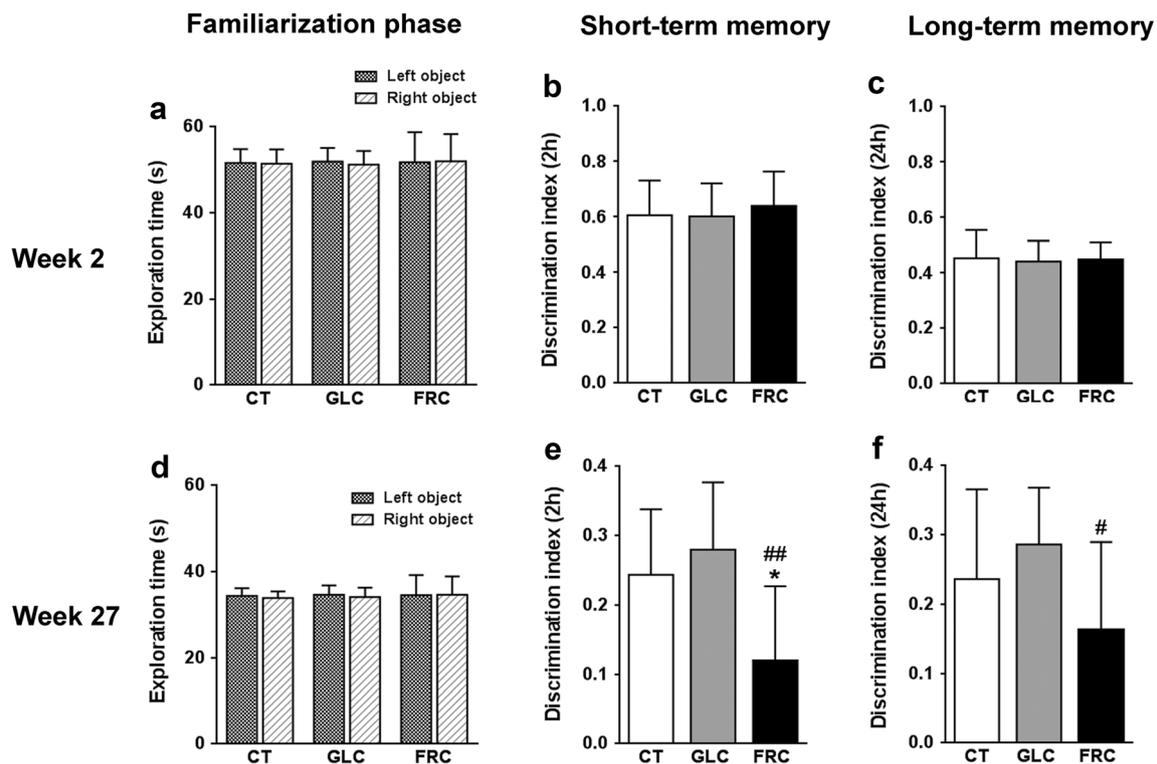


Fig. 2 Results of the Novel Object Recognition (NOR) Test. The bars show the performance of control (CT), Glucose- (GLC) and fructose-supplemented (FRC) rats during the training phase (a, d) and in the test performed 2 h (short-term memory) and 24 h (long-term memory) after the training phase, at week 2 (b, c) and week 27 (e, f) after the

commencement of their respective diets. The discrimination index (DI) was defined as $(TN - TO)/(TN + TO)$, being TN and TO the time spent by each rat exploring the novel object and the old object, respectively. Results are the mean \pm SD of values from 11 to 12 animals/group. * $p < 0.05$ vs CT; # $p < 0.05$, ## $p < 0.01$ vs GLC

Sugar Supplementation Does Not Induce Neuroinflammation in the Frontal Cortex or the Hippocampus

Neuroinflammation is one of the mechanisms that may underlie cerebral insulin resistance. However, the mRNA expression of pro-inflammatory molecules tumor necrosis factor (*tnf*) α , monocyte chemotactic protein (*mcp*)-1, and interleukin (*il*)-6 were not significantly modified after sugar supplementation in either the frontal cortex or the hippocampus (Fig. 6a). Moreover, the immunohistochemical assessment of glial fibrillary acidic protein (GFAP) expression, a marker of astroglial activation, did not reveal any differences between groups in the frontal cortex or the hippocampus (Fig. 6b, c).

Effects of Fructose Supplementation on Oxidative Stress and Markers of Mitochondrial Dynamics

Increased production of reactive oxygen species (ROS) and oxidative stress have also been related to the establishment of insulin resistance. We examined the mRNA expression of glutathione peroxidase (*gpx1*), a major peroxide-scavenging enzyme, superoxide dismutase (*sod2*), which converts superoxide into hydrogen peroxide, and catalase (*cat*), which is responsible for the decomposition of hydrogen peroxide to

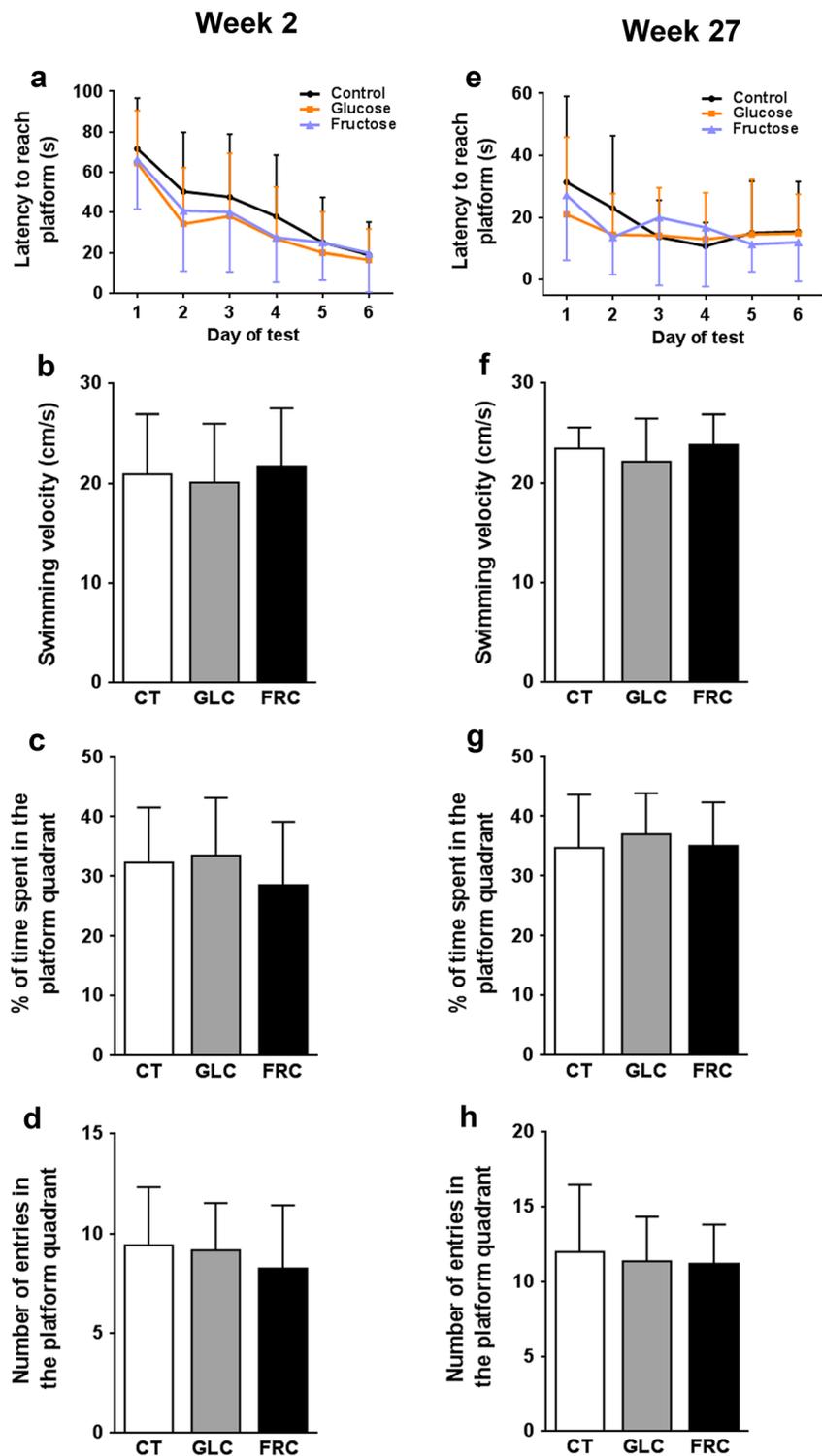
water and oxygen. Our results showed that the mRNA levels of *gpx1*, *sod2*, and *cat* in the frontal cortex of fructose-supplemented rats were significantly reduced; however, in the hippocampus, only *sod2* mRNA expression decreased significantly in the fructose group (Fig. 7a).

As mitochondrial dysfunction has also been related to impaired neuronal function, the expression of mitochondria-shaping proteins optic atrophy 1 (*Opa1*) and mitofusin 2 (*Mfn-2*), which promote mitochondrial fusion, and dynamin-related protein 1 (*Drp1*), which participates in mitochondrial fission, was determined. As shown in Fig. 7b, fructose supplementation significantly reduced *Opa1* expression in the frontal cortex, whereas it increased the expression of *Drp1*, which suggests impaired mitochondrial dynamics. By contrast, glucose supplementation did not alter the expression of these proteins significantly. None of these proteins was affected by sugar supplementation in the hippocampus (Fig. 7c).

Cognitive Alterations Are Associated with Metabolic Changes

A partial correlation analysis was performed in order to determine the relationship between the DI (the parameter used to assess the performance of the rats in the NOR test) and several metabolic and molecular parameters that may

Fig. 3 Evaluation of spatial learning and memory performance of control (CT), Glucose- (GLC) and fructose-supplemented (FRC) rats using the Morris Water Maze (MWM) Test at week 2 (**a–d**) and week 27 (**e–h**) after the commencement of their respective diets. The graphs show the escape latency time to reach the hidden platform during training days (**a, e**) and the swimming velocity (**b, f**), the percentage of time spent in the platform zone (**c, g**), and the number of entries in the platform zone (**d, h**) during the probe trial. Results are the mean \pm SD of values from 11 to 12 animals/group



be related to key events in neurodegeneration processes, after 7 months of sugar supplementation (Supplementary Table 2 and Fig. 8). The most interesting findings were a significant negative correlation between the DI and plasma triglyceride values ($r = -0.650$, $p < 0.001$) and a positive correlation between triglyceride and insulin plasma levels ($r = 0.493$, $p < 0.05$), which suggests that

hypertriglyceridemia may contribute to the cognitive impairment and also to the hyperinsulinemia observed in fructose-supplemented rats. Moreover, we found significant correlations between the DI and the expression of molecules related to oxidative stress (Sod2: $r = 0.462$, $p < 0.05$) and mitochondrial dynamics (Opa1: $r = -0.877$, $p < 0.001$ and Drp1: $r = 0.825$, $p < 0.001$) in the frontal cortex.

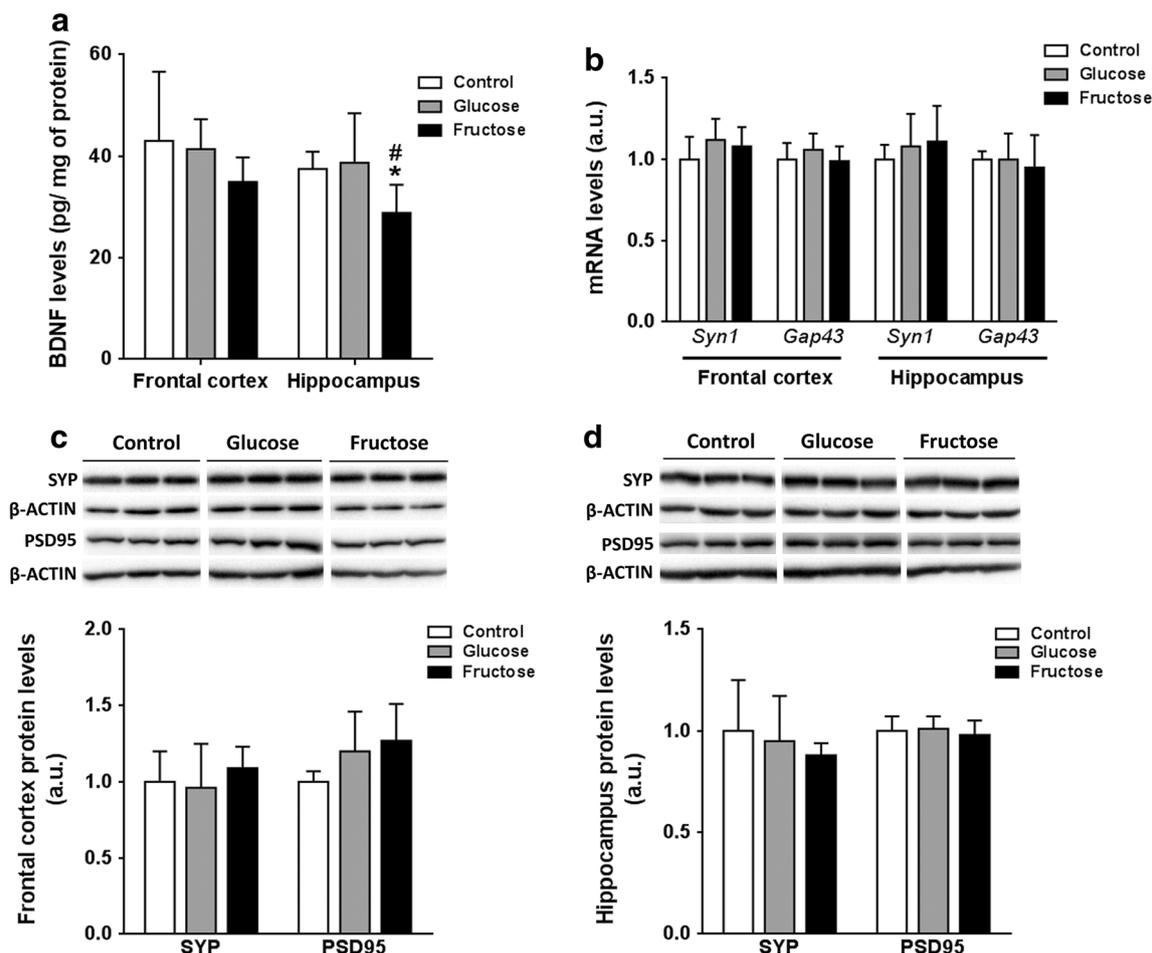


Fig. 4 **a** Levels of BDNF in the frontal cortex and in the hippocampus of control and sugar-supplemented rats determined by ELISA test. **b** mRNA levels of *syn1* and *gap43* in the frontal cortex and in the hippocampus of control and sugar-supplemented rats ($n = 8/\text{group}$). Western blot of SYP and PSD95 in samples from the frontal cortex (**c**) and the hippocampus

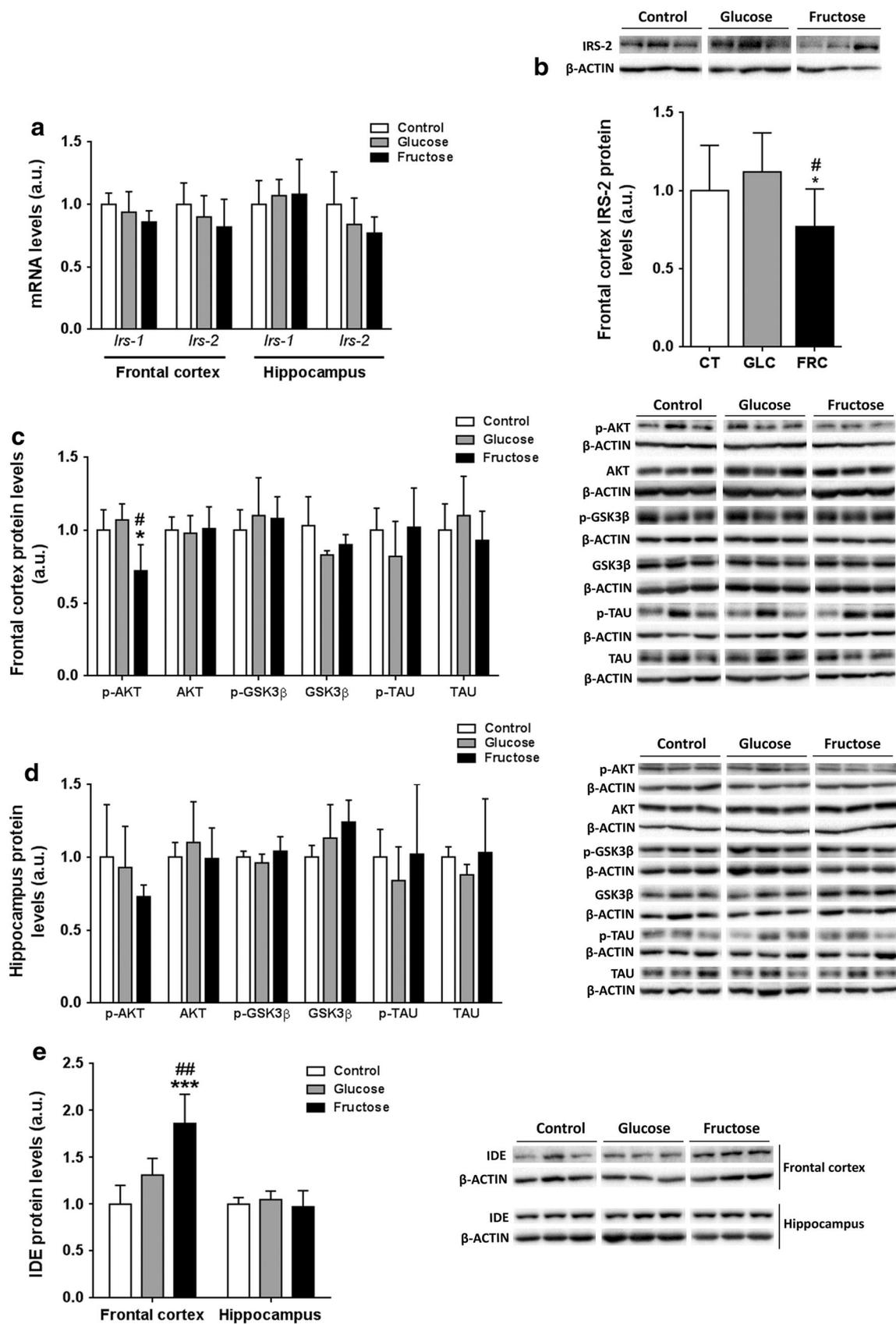
(**d**) obtained from control, glucose- and fructose-supplemented rats. Representative bands corresponding to 3 different rats in each group are shown; bar plots show the level of the proteins expressed as the mean (a.u., arbitrary units) \pm SD of the values obtained from four to five animals. * $p < 0.05$ vs control values; # $p < 0.05$ vs glucose group values

Discussion

Diet-induced cognitive deficits and their relationship with metabolic alterations have been the subject of extensive research, but it is still a matter of debate whether these effects depend exclusively on the high amount of energy provided by the diets or are related to specific nutrients. Our results support the latter, as they show that long-term exposure to a diet supplemented with liquid fructose in female rats causes metabolic and cognitive alterations, whereas consumption of an isocaloric supplement of liquid glucose does not. Specifically, only fructose consumption alters the results of the GTT and impairs brain insulin signaling, while reducing the rats' performance in the NOR test. Moreover, only fructose-supplemented rats exhibit increased plasma levels of triglycerides, AGE, and TBARS, as well as reduced expression of antioxidant enzymes and altered mitochondrial dynamics in the frontal cortex.

In humans, some reports suggest impaired postprandial memory after consumption of a meal with a high glycemic index [23–25], whereas others show that drinking glucose can temporarily enhance cognitive abilities [26, 27]. By contrast, our results did not show any differences in cognitive performance in the NOR test after short-term sugar supplementation (2 weeks), but when the sugar supplementation period was extended to 27 weeks memory deficits were observed, only in animals that consumed fructose. Surprisingly, total

Fig. 5 **a** mRNA levels of IRS-1 and -2 in the frontal cortex and in the hippocampus of control and sugar-supplemented rats ($n = 8/\text{group}$). Western blot of IRS-2 (**b**), phosphorylated and total AKT, GSK3 β and TAU (**c, d**) and IDE (**e**) in samples from frontal cortex and hippocampus obtained from control, glucose- and fructose-supplemented rats. Representative bands corresponding to three different rats in each group are shown; bar plots show the level of the proteins expressed as the mean (a.u., arbitrary units) \pm SD of the values obtained from four to five animals. * $p < 0.05$, *** $p < 0.001$ vs control values; # $p < 0.05$, ## $p < 0.01$ vs glucose group values



exploration time during the familiarization phase of the NOR test was higher at week 2 than at week 27, although there were no differences between the three dietary groups. The reduction in exploratory behavior at week 27 is probably a consequence of the habituation to the open field and the reduction of curiosity, and may be responsible for the lower DI values, compared to week 2. Despite this, the total exploration time was enough to evaluate the cognitive status in the test phase, and our results clearly show a reduced DI after 27 weeks of fructose supplementation.

Most studies concerning the effects of dietary components on cognitive abilities in animal models use only one type of test, but it has been suggested that memory impairments after simple sugar consumption may differ depending on the cognitive test used [28]. Our results show that, in contrast to the NOR test results, learning and memory assessed through the MWM test were not impaired by sugar supplementation. The NOR test is based on the rodents' spontaneous preference for novelty, and it is a widely used model for researching memory alterations through the evaluation of the animals' ability to recognize a novel object [29]. The MWM test, on the other hand, evaluates spatial working memory and learning, and is considered useful for assessing damage to the hippocampus [30]. Hippocampal tissue seems to play a more important role in spatial memory than in non-spatial memory [31], which is evaluated more effectively in the NOR test. In most studies, the NOR task does not seem to be impaired by hippocampal lesions [32]. Thus, the memory deficits observed in our study, in the NOR test alone, suggest that the main effect of fructose supplementation does not take place in the hippocampus. However, the involvement of this tissue in the cognitive alterations observed cannot be ruled out entirely. A combination of the NOR test with other tests, such as the place recognition test, which has been reported to be dependent on the hippocampus [8], could help to discriminate between hippocampal- and non-hippocampal-mediated memory. Moreover, it has been shown that with retention intervals of less than 15 min in the NOR test the cortex is sufficient for object recognition, but with longer intervals (as in the present study, i.e., 2 and 24 h), the hippocampus is also required [31]. In addition, the sex of the animals may influence the rats' performance in a given test. For example, cognitive deficits in the spatial water maze test were observed in male rats supplemented with high-fructose diets for four to 5 months [33, 34], but not in female rats [35]. This suggests that estrogens may be protective against the development of these alterations [9]. Along these lines, Abbott et al. [36] showed that female rats were capable to correctly perform a test dependent on hippocampal function, place recognition, when they were in the high-estrogen (proestrus) phase, but not during the low-estrogen (metestrus phase). Thus, we cannot exclude the possibility that estrous phase of the rats precluded an effect on hippocampal-related memory.

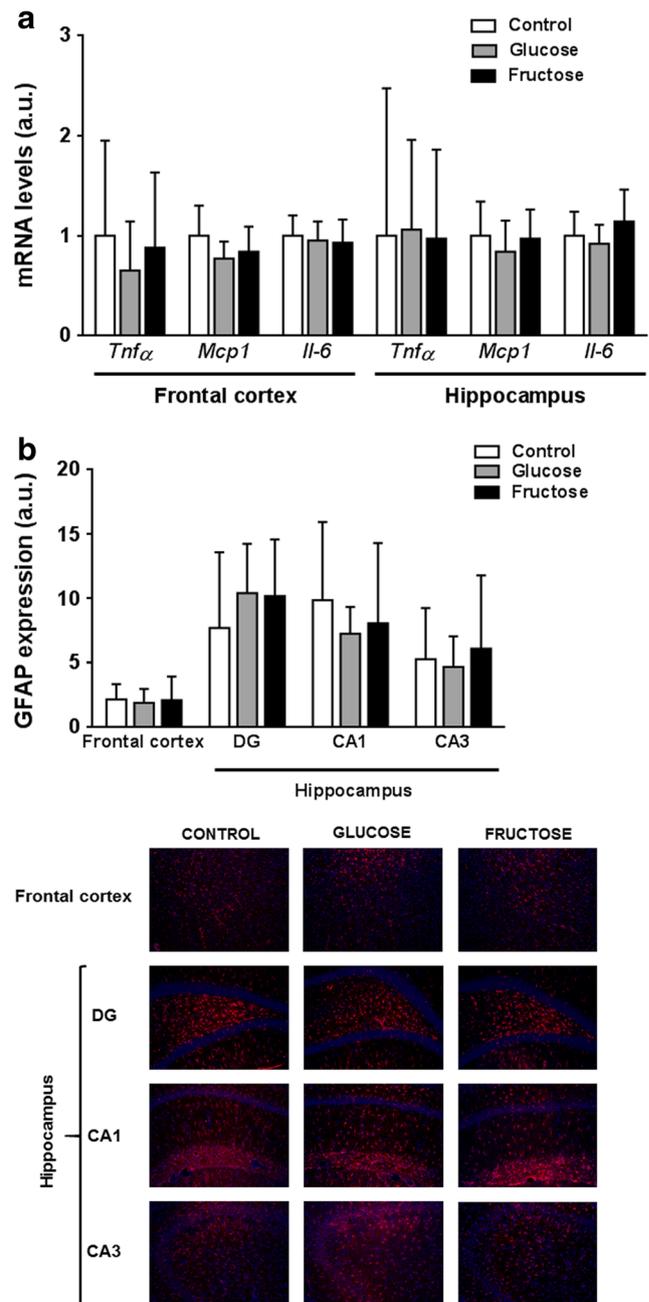
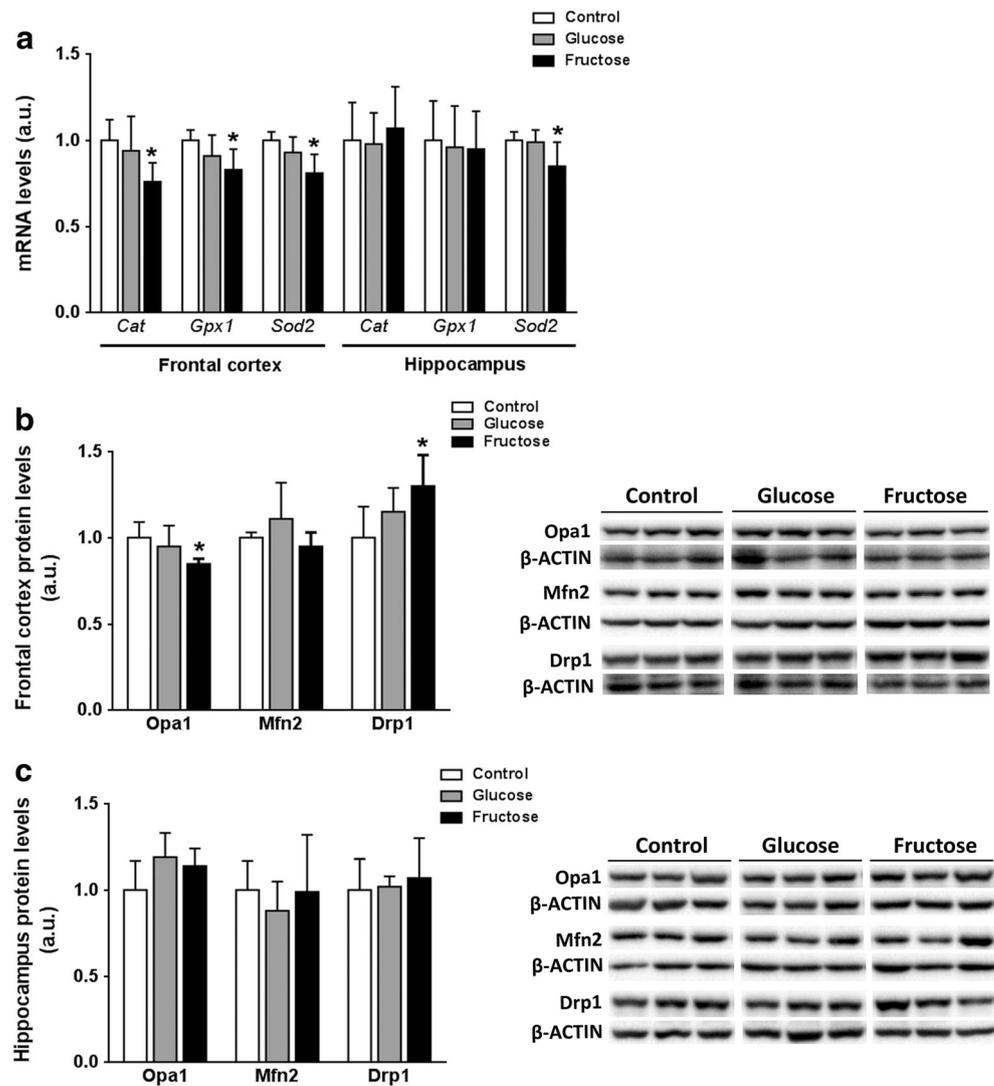


Fig. 6 **a** Bar plots showing the relative levels of specific mRNAs of pro-inflammatory molecules in the frontal cortex and in the hippocampus of control and sugar-supplemented rats ($n = 8/\text{group}$). **b** Quantification and representative images for GFAP immunostaining in control, fructose- and glucose-supplemented rats. Bars represent the mean (a.u., arbitrary units) \pm SD of the values obtained from four to five animals/group. CA Cornu Amonis, DG Dentate gyrus

In that regard, the levels of BDNF protein, a neurotrophic factor that plays an important role in synaptic plasticity, were found to be lower in both the frontal cortex and the hippocampus, and the reduction was more intense and significant in the hippocampus. Although it has been reported that BDNF levels are regulated by excessive dietary energy [37], our results clearly show a specific reduction in fructose-supplemented

Fig. 7 **a** Bar plots showing the relative levels of specific mRNAs of oxidative stress-related molecules in the frontal cortex and in the hippocampus of control and sugar-supplemented rats ($n = 8/\text{group}$). Western blot of proteins involved in mitochondrial dynamics in the frontal cortex (**b**) and hippocampus (**c**) obtained from control, glucose- and fructose-supplemented rats. Representative bands corresponding to three different rats in each group are shown; bar plots show the level of the proteins expressed as the mean (a.u., arbitrary units) \pm SD of the values obtained from four to five animals. $*p < 0.05$ vs control values

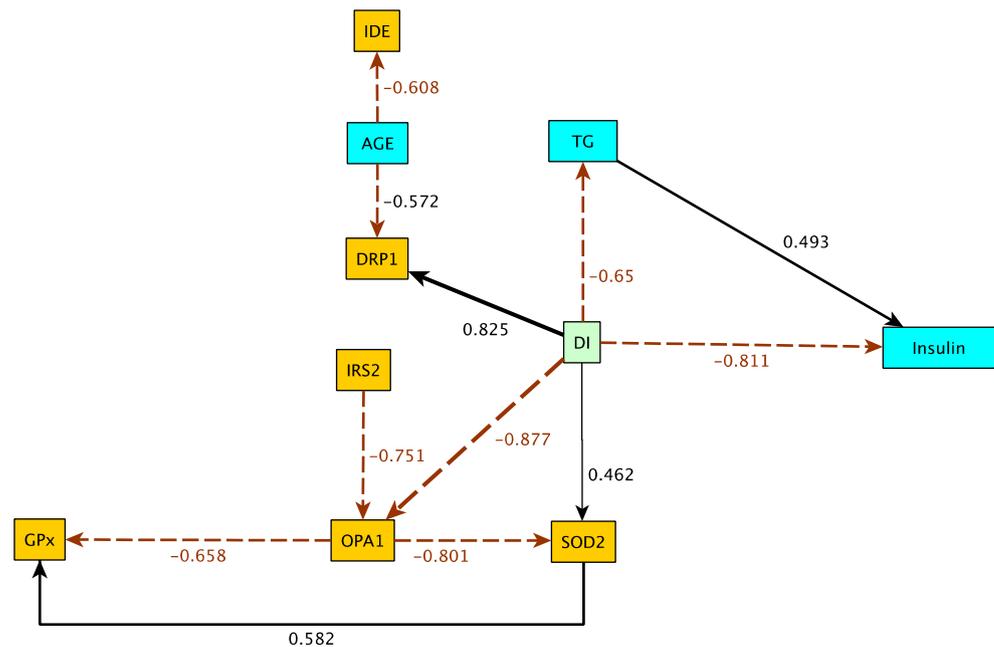


rats, despite the fact that the energy intake was identical in both sugar-supplemented groups. Several studies in laboratory animals suggest that diets enriched with sugars and fats lead to a reduction in BDNF levels that correlates with cognitive decline [37, 38]. In humans, reduced BDNF expression is already evident in preclinical phases of Alzheimer's disease (AD) [31]. Thus, the observed reduction in hippocampal BDNF may be an early marker of dysfunction that appears before specific hippocampus-related cognitive deficits are apparent. Moreover, our results show that downstream effectors of the BDNF pathway (SYN1 and GAP43) are not affected in sugar-supplemented rats. Thus, it is plausible that longer supplementation with fructose could lead to further impairment of BDNF signaling, thereby resulting in memory deficits in the MWM test.

One key observation of the present study is that, despite being isocaloric, fructose supplements caused cognitive alterations, while glucose supplements did not. This is consistent with the presence of metabolic alterations in fructose-

supplemented rats only, which reveals the mechanism underlying the cognitive deficits. On the one hand, rats supplemented with fructose present clear hypertriglyceridemia, which is not observed in glucose-supplemented rats, and there is a strong negative correlation between the DI obtained in the NOR test and the plasma triglyceride levels after 7 months of sugar supplementation (Supplementary Table 2, Fig. 8). Thus, our results are consistent with studies showing that high triglyceride levels can cause cognitive alterations [39–41]. On the other hand, only rats that received fructose present significant increases in plasma insulin levels, under fasting conditions and throughout the GTT, which indicates that the rats may secrete more insulin in order to maintain normal blood glucose levels. Accordingly, fructose-drinking rats show a reduction in whole-body insulin sensitivity, assessed by the ISI, which suggests a state of IR. Studies in hamsters also show that fructose induces an IR state, together with a considerable increase in VLDL-triglyceride production, and suggest that the culprit for these effects is not fructose per se but the IR

Fig. 8 Behavioral, plasma level and neuronal markers hierarchical network of the three rat groups ($n = 36$) obtained by using yEd graph editor (v. 3.14.4). Each node represents one behavioral, plasma level or neuronal marker and each edge between two nodes represents the partial correlation. Colors represent the different variables and node dimensions represent the number of correlations. Solid black line represents positive correlation and dotted red line represents negative correlation



caused by fructose [42]. In this regard, a significant positive correlation was observed between plasma triglyceride and insulin levels (Supplementary Table 2, Fig. 8). Moreover, IR is a hallmark of T2D, and clinical studies have linked pre-diabetic IR states and T2D to cognitive dysfunctions and dementia [2]. It has been suggested that peripheral IR is accompanied by central IR, which may be a mechanistic mediator of cognitive deficits [2, 43]. In the present study, we observed that fructose-but not glucose-supplemented rats show insulin-signaling impairment in the brain, once again suggesting that the effect is specifically related to fructose rather than increased calorie intake. A hippocampal insulin-signaling defect has previously been observed in fructose-fed rats or hamsters [33, 44, 45]. Our results are consistent with these reports, as they show a trend towards reduced Akt phosphorylation in the hippocampus in the context of increased plasma insulin, a situation in which activation of Akt would be expected. Most studies concerning diet-induced central IR have focused on the hippocampus, as this tissue expresses high levels of insulin receptors [46] and is particularly sensitive to changes in lifestyle factors, such as diet [37, 47]. However, all components of the insulin-signaling cascade are also expressed to a comparable extent in the cortex [48], and dietary changes may also affect this brain region. Indeed, we examined insulin signaling in the frontal cortex also, and observed a clear impairment, revealed by significantly reduced IRS-2 protein levels and Akt phosphorylation. It could be hypothesized that the memory deficit observed in the NOR task, which as commented before depends less on the hippocampus than the cortex, may be related to the more intense insulin-signaling impairment in this brain region. These results highlight the importance of the cortex, which has received less attention than the hippocampus in past

studies, in the establishment of cognitive deficits induced by fructose supplementation.

IR has been associated with decreased IDE levels [22, 49]; by contrast, we observed an increase in IDE protein expression in fructose-drinking rats. Insulin has been shown to regulate IDE expression [50], and the hyperinsulinemia presented by the fructose group could therefore explain this increase. Alternatively, increased IDE may be regarded as a compensatory mechanism to reduce high insulin levels due to its insulin-degrading activity. Some reports suggest an inverse correlation between plasma and cerebrospinal fluid insulin in AD patients [48]. Thus, although brain insulin levels were not measured in this study, it could be hypothesized that increased IDE expression in fructose-supplemented rats leads to reduced insulin concentrations in the brain, thereby contributing to impaired insulin-signaling and cognitive deficits.

Other factors, such as inflammation and oxidative stress, may play a key role in cognitive dysfunctions, either directly or through IR-related mechanisms [2]. Our results, which show unaltered expression of the main inflammatory mediators and astrogliosis markers, rule out the participation of neuroinflammation in our model. In contrast, Cigliano et al. recently reported hippocampal inflammation in young and adult male rats after short-term consumption of fructose in solid form [45], and suggested that inflammation may be linked to increased intestinal permeability and microbiota remodeling [45, 51]. The difference between our results and those from Cigliano et al. may lie in the way the body handles solid compared to liquid fructose; thus, we have previously shown that feeding mice with a Western-type solid diet as a source of fructose increases intestinal permeability to bacterial toxins, whereas liquid fructose supplementation (15% w/v) does not

[52]. The fact that we used females in our study, whereas Cigliano et al. used males, may have also contributed to the lack of effect of dietary sugars on neuroinflammation. In this sense, Abassi et al. recently reported sex-differences in the activation of inflammatory pathways, and specifically in those related to brain function [53].

On the other hand, plasma AGE levels are selectively increased in fructose-drinking rats (Table 1), thus highlighting a role for oxidative stress. In this regard, the formation of AGEs (irreversible adducts derived from the non-enzymatic reaction between sugars, such as glucose and fructose, and proteins) increases under oxidative conditions [54] and, in a positive feedback loop, AGEs may increase the formation of reactive oxygen species [55] and down-regulate intracellular antioxidant systems, such as SOD and glutathione [56]. In this regard, a reduction in the mRNA expression of antioxidant enzymes in the frontal cortex of fructose-supplemented rats was observed, and this may contribute to increased oxidative stress. Accordingly, plasma TBARS levels, a marker of lipid peroxidation that occurs as a consequence of oxidative imbalance [57], increase only in the fructose group. The fact that both AGE and TBARS are selectively increased by fructose but not glucose supplementation may derive from fructose-specific mechanisms, such as its higher reactivity to form AGE compared with glucose [58]. Moreover, an increase in TBARS may result from fructose-increased triglyceride levels, as serum triglycerides have been shown to positively correlate with the amount of TBARS [59].

Oxidative stress-related neurodegenerative diseases, such as AD and age-related cognitive disorders, are also characterized by dysfunctions in mitochondrial dynamics [60, 61]. Specifically, excessive mitochondrial fission resulting in fragmented mitochondria is an early event in the progression of these pathologies [62] and has also been observed in the neurons of diabetic mice [63]. Our results also show an increase in the expression of the fission-related protein Drp1 and a decrease in Opa1, which is involved in the fusion process, in the frontal cortex of fructose-drinking rats. Although mild, these changes suggest that fructose induces an imbalance in mitochondrial dynamics in this area of the brain that may result from increased oxidative stress and can further contribute to it, in a vicious cycle leading to the progression of cognitive dysfunction. Finally, the hierarchical network created by partial correlation analysis clarifies the molecular mechanisms underlying the cognitive impairment through diet consumption. In addition, the network shows the five relevant nodes directly associated with cognition, namely insulin plasma levels, TG plasma levels, and OPA1 expression, with negative effects, and DRP1 and SOD2 expression, with positive effects.

In conclusion, our results show that fructose supplementation in liquid form induces cognitive deficits in female rats that are strongly associated with metabolic alterations, specifically

hypertriglyceridemia, hyperinsulinemia, and changes in molecular markers of oxidative stress and mitochondrial dynamics. These alterations, which are absent in isocaloric glucose-supplemented rats and are therefore not exclusively related to the increased calorie intake, could underlie defects in molecular pathways, such as brain insulin signaling, and lead to cognitive impairment. Given the lack of effects of glucose, we could speculate that the observed effects of fructose might not occur, or might be less intense, when combined with glucose (in HFCS or sucrose), as it is commonly consumed in SSB.

Acknowledgements We are a Consolidated Research Group of the Autonomous Government of Catalonia (SGR13-00066). We would like to thank the University of Barcelona's Language Advisory Service for revising the manuscript.

Funding Information This study was supported by the Fundació Privada Catalana de Nutrició i Lípids, Ministry of Economy and Competitiveness (grant number SAF2013-42982-R) and European Commission FEDER funds. Miguel Baena and Gemma Sangüesa were supported by FPI and FPU grants from the Spanish Ministry of Science and Innovation.

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