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High-Fructose Consumption Impairs the Redox System and Protein Quality Control in the Brain of Syrian Hamsters: Therapeutic Effects of Melatonin

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Abstract
Although numerous studies have demonstrated the harmful effect of excessive fructose consumption at the systemic level, there is little information on its effects in the central nervous system. The purpose of the present work was to study the cellular alterations related to oxidative stress and protein quality control systems induced by a high-fructose diet in the brain of Syrian hamsters and their possible attenuation by exogenous melatonin. High-fructose intake induced type II diabetes together with oxidative damage, led to alterations of the unfolded protein response by activating the eIF2α branch, and impaired the macroautophagic machinery in the brain, favoring the accumulation of aggregates labeled for selective degradation and neurodegeneration markers such as β-amyloid (1–42), tau-p-S199, and tau-p-S404. Melatonin attenuated the manifestation of type II diabetes and reduced oxidative stress, deactivated eIF2α, and decreased tau-p-S404 levels in the brain of animals fed a high-fructose diet.

Keywords High-fructose · Brain · ER stress · Autophagy · Neurodegeneration · Melatonin

Introduction

Currently, obesity and diabetes mellitus have become a public health problem with a prevalence that is increasing at an alarming rate. Several hypotheses have been proposed to explain this phenomenon, such as lifestyle changes characterized by an increase in the consumption of processed foods and sugar-sweetened beverages, the main ingredients of which are usually sucrose or fructose corn syrup, coupled with reduced physical activity [1]. Recently, the World Health Organization (WHO) has recommended the consumption of free sugars, such as fructose and glucose, to not exceed 10% of the total daily caloric intake [2].

Fructose is a monosaccharide with the same empirical formula as glucose, but with a different structure. Fructose is usually found in its free form in fruits, vegetables, and honey or forms a disaccharide with glucose called sucrose or common sugar. Although fructose is incorporated into glycolysis at different levels, the metabolism of both monosaccharides is different. Fructose is preferentially metabolized in the liver, while glucose is mainly metabolized in the brain [3]. Thus, although many studies have already demonstrated the harmful effect of excessive consumption of fructose at the multiorgan level, at present, there are few studies on its effects at the level of the central nervous system (CNS).

Although fructose was initially used as a sweetener for diabetics because of its low glycemic index, clinical studies have shown that excessive consumption of fructose can lead to metabolic complications, such as type 2 diabetes, insulin resistance (IR), obesity, and major lactate production and lipid oxidation [1]. Due to these effects, several studies have correlated IR caused by high-fructose intake with an increased risk of neurodegenerative diseases, such as dementia [4, 5]. In fact, the relationship between IR in the brain and the development

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of Alzheimer's disease (AD) was recently identified [6–9], and studies on memory and cognition note an association between fructose consumption and cognitive impairment [10]. In addition, high and continued consumption of fructose has been shown to induce neuroinflammation and oxidative stress in the brain, both of which are involved in the pathogenesis of neurodegenerative diseases [11].

Inflammation and oxidative stress particularly affect the brain by inducing morphological and functional changes associated with alterations of neuronal processes, such as synaptic function and plasticity, signal neurotransmission and metabolism, which ultimately lead to alterations in learning and memory [12]. The susceptibility of the brain to these neurobiological alterations induced by oxidative stress may be due to its high oxygen consumption, high energy demand, high abundance of polyunsaturated fatty acids and lipids, and relatively low antioxidant capacity [13]. It has been observed that increased oxidative stress in the brain can lead to the accumulation of misfolded proteins such as α-synuclein in Parkinson's disease (PD) and β-amyloid protein in AD, and even a deterioration in the mechanisms of protein degradation [14].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a pleiotropic neurohormone that is mainly produced by the pineal gland from tryptophan and controls various physiological processes associated with day-night cycles. Synthesis and release of melatonin into the bloodstream are regulated by exposure to dark stimulation, helping to synchronize circadian rhythms with light-dark cycles. In addition to its chronobiotic properties and ability to influence the neuroendocrine-reproductive axis that controls seasonal reproduction, melatonin also has important antioxidant properties. It is considered as one of the best natural antioxidants, acting directly as a free radical scavenger or indirectly by stimulating the gene expression and activity of antioxidant enzymes [15]. Due to this diverse range of physiological effects, therapeutic application of melatonin could neutralize the damage associated with obesity and neurodegenerative diseases [16].

Syrian golden hamsters are small rodents that have many features that resemble human physiology, such as diet and metabolism [17, 18]. They are obesity prone and develop insulin resistance when fed a high-fat/high-carbohydrate diet [19, 20]. Unlike rats and mice, hamsters develop hypercholesterolemia and hypertriglyceridemia when fed fat- and cholesterol-rich diets. Furthermore, they have cardiovascular and hepatic systems similar to those of humans [21] and can thus be a useful model for studying diet-induced alterations [22–25].

Taking into account the limited information available regarding the effects of high carbohydrate diets, especially in the form of high-fructose, at the level of the CNS, the main objective of the present work was to study the cellular alterations related to oxidative stress and protein quality control systems in the brain of Syrian hamsters fed a high-fructose diet to identify potential therapeutic targets. We also tested their possible attenuation by exogenous administration of melatonin.

Materials and Methods

Animals and Reagents

Sixteen 8-week-old male Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Barcelona, Spain). Animals were housed two per cage in the vivarium of the University of Oviedo under a 14:10 h dark-light cycle at 22 ± 2 °C and received tap water and food ad libitum. After a 2-week acclimatization period, the animals were randomly divided into four experimental groups with four mice per group ($n = 4$) as follows: normal diet (ND)—hamsters from this group received a normal diet for rodents with macronutrient composition of 14.3% protein, 48% carbohydrate, and 4% fat (Teklad 2014 Global Rodent Maintenance Diet); normal diet + melatonin (ND + M)—hamsters from this group received a normal diet and a daily dose of 500 μg melatonin per kilogram body weight in a saline solution with 0.5% ethanol administered via a subcutaneous injection between the shoulder blades; fructose diet (FD)—hamsters in this group received a high-fructose (60%) diet for rodents with composition of macronutrients of 18.3% protein, 60.4% carbohydrate, and 5.2% fat (TD89247, Teklad); and Fructose diet + melatonin (FD + M)—hamsters in this group received a high-fructose diet and a daily dose of 500 μg melatonin per kilogram body weight in a saline solution with 0.5% ethanol administered via a subcutaneous injection between the shoulder blades. The macronutrient energy ratio in both types of diet is divided as follows: 20% of calories come from proteins, 67% of calories are from carbohydrates, and 13% of calories are from fats.

The experiment was carried out for 10 weeks, and melatonin was administered daily to the ND + M and FD + M groups half an hour after lights off (ZT14.5). Thus, melatonin administration coincided with the onset of the nocturnal melatonin peak. The ND and FD groups received vehicle (0.5% ethanol: saline in proportion to its body weight). After the respective treatments, hamsters were fasted for 24 h before sacrifice by decapitation, blood samples were collected, and the brains were dissected, frozen, and stored at − 80 °C until further use.

Body and Blood Parameters

Body weight was recorded at the beginning and at end of the experiment, and food intake was measured per cage twice weekly. Brain weight was also recorded, and the blood parameters (glucose, insulin, HDL cholesterol, LDL cholesterol, and

uric acid) were analyzed by routine laboratory tests at the Laboratory of Veterinary Analysis Dr. Barba (Madrid, Spain).

Tissue Processing and Protein Quantification

The brain of each hamster was homogenized in RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.5% doxycholate, 1% NP40, 0.1% SDS, 1 mM PMSF). The homogenates were centrifuged at 900×*g* for 6 min at 4 °C. Supernatants containing proteins were collected, aliquoted, and frozen at − 80 °C until further analysis. The Bradford method was used to quantify the protein concentrations of brain homogenates [26].

Lipid Peroxidation (LPO)

Malondialdehyde (MDA) and 4-hydroxyalkenes, such as 4-hydroxy-2(E)-nonenal (4-HNE), are end products derived from the peroxidation of polyunsaturated fatty acids and related esters and provide an adequate index of oxidative damage to lipids [27]. For LPO determination, we used the 1-methyl-2-phenylindole colorimetric method (586 nm) [28]. The results are expressed as micromole MDA + 4-HNE per gram protein.

Superoxide Dismutase and Catalase Activities

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined from the protocol of Martín et al. [29]. This enzyme inhibits hematoxylin auto-oxidation to the colored compound hematein, which absorbs at 560 nm. The results are expressed as enzymatic units per milligram protein, taking into an account that one SOD unit is equivalent to 0.039 absorbance units. Catalase (CAT; EC 1.11.1.6) activity was assayed using the method from Lubinsky and Bewley [30] using H_2O_2 as the substrate. Disappearance of the substrate was measured by spectrophotometry (240 nm). The results are expressed as micromole H_2O_2 per milligram protein per minute.

Total Antioxidant Capacity

The total antioxidant capacity (TAC) was assessed by a modification [31] of the 2,2′-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{\cdot+}$) cation radical method [32]. The decay of the $ABTS^{\cdot+}$ radical was measured at 730 nm. The results are expressed as milligram Trolox equivalents per milligram protein.

20S Proteasome Activity

The activity of the 20S proteasome was assessed using a 20S proteasome activity assay kit (APT280; Chemicon, Merck Millipore, Billerica, MA, USA) based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after its cleavage from the labeled substrate LLVY-AMC by the chymotrypsin-like activity of the proteasome. Free AMC was detected by fluorometric quantification (380/460 nm). The results are presented as micromolar AMC per milligram protein.

Western Blot Immunoassay

Tissue homogenates (100 μg of protein per sample) were mixed with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) and denatured by boiling at 100 °C for 5 min. The samples were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes at 350 mA (Immobilon TM-P; Millipore Corp., Bedford, MA, USA).

The membranes were blocked with 5 or 10% (*w*/*v*) nonfat dry milk dissolved in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. Subsequently, membranes were incubated with the following primary antibodies overnight at 4 °C: anti-IRE1α (3294), anti-phosphorylated-eIF2α (3398), anti-ubiquitin (3933), anti-β-amyloid 1–42 (14974), and anti-α-synuclein (2642) from Cell Signaling Technology (Danvers, MA, USA); anti-ATF-6α (sc-22,799), anti-cathepsin D (sc-6486), and anti-beclin-1 (sc-10086) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); tau phosphorylation site-specific antibodies p-S199, p-T205, p-S396, and p-S404 (44779G) from Invitrogen (Waltham, MA, USA); anti-LAMP2A (ab18528) from Abcam (Cambridge, UK); anti-LC3 (PD014) from MBL (Naka-ku Nagoya, Japan); and anti-p62 (H00008878-M01) from Abnova (Walnut, CA, USA), each previously diluted 1:1000 in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% (*w*/*v*) nonfat dry milk and 0.02% sodium azide. After three 10-min washes in TBS-T, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000 in TBS containing 1% (*w*/*v*) nonfat dry milk for 1 h at room temperature, followed by three 10-min washes in TBS-T.

The membranes were developed using a chemiluminescent substrate (WBKLS0500, Merck Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The protein levels were quantitated using Image Studio Lite 5.2.5 software (LI-COR Biotechnology, Lincoln, NE, USA). The results were normalized to Ponceau S and are expressed as a percentage of the experimental group ND.

Statistical Analysis

All of the results are presented as the mean values ± standard deviations (SD) of the means, derived from at least three separate experiments. The results were analyzed by bidirectional

analysis of variance (ANOVA) to study the effects of diet and treatment with melatonin, followed by a Bonferroni post hoc test. Differences were considered statistically significant when $p < 0.05$. Statistical analyses and histograms were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

Results

High-Fructose Diet Alters Glucose and Lipids Homeostasis

Although there were no significant differences in food intake, body weight gain, or brain weight at the end of the study (Table 1), we found obvious changes in the blood biochemistry. In comparison with animals fed the ND, hamsters subjected to the FD developed higher levels of blood glucose ($p < 0.001$) (Fig. 1a), but no changes in insulin levels. In addition, melatonin treatment increased the insulin level in FD hamsters, whereas the glucose level was reduced ($p < 0.001$) to the level found in the ND group (Fig. 1a, b). Furthermore, although we did not observe differences in the high-density lipoprotein cholesterol (HDL) concentration, FD hamsters showed higher levels of low-density lipoprotein cholesterol (LDL) ($p < 0.01$) than ND hamsters that were normalized by treatment with melatonin ($p < 0.01$) (Fig. 1d). Finally, the uric acid levels, which showed no differences between ND and FD animals, decreased in the ND + M ($p < 0.001$) and FD + M groups ($p < 0.05$) (Fig. 1e).

High-Fructose Diet Induces Oxidative Stress in the Brain

To determine whether the high-fructose diet caused cellular alterations in the brain, we measured markers of oxidative damage and antioxidant defense in brain homogenates. Thus, FD hamsters showed higher LPO in the brain ($p < 0.05$) than ND animals. Melatonin administration to ND hamsters resulted in no changes in LPO, whereas LPO significantly decreased in the FD + M group ($p < 0.05$) (Fig. 2a). To evaluate the antioxidant status in brains from the four experimental groups, we determined the activity of antioxidant enzymes, SOD and CAT, and the total antioxidant capacity (TAC), which includes both enzymatic and non-enzymatic antioxidants. The three determinations were higher in FD animals ($p < 0.001$) (Fig. 2b–d). Although melatonin treatment was able to counteract LPO in brains from FD animals, the SOD and CAT activities remained stable, while the TAC was reduced ($p < 0.001$) (Fig. 2d).

High-Fructose Adapts the Unfolded Protein Response

In many pathological situations, oxidative stress coexists with endoplasmic reticulum (ER) stress, favoring the accumulation of misfolded proteins. To study the possible presence of ER stress in the brains of hamsters fed with fructose, we measured the content of the key proteins involved in the activation of the three arms of the unfolded protein response (UPR): IRE1α, ATF-6α, and eIF2α. We did not observe significant differences in the protein expression of (IRE1α), a protein responsible for monitoring ER homeostasis. Surprisingly, we found lower protein levels of activating transcription factor 6 in its active form (50 kDa-ATF-6α) in FD than in ND animals ($p < 0.05$), showing no differences compared with the respective melatonin-treated group (Fig. 3). However, analysis of the eukaryotic-2α initiation factor (eIF2α) in its phosphorylated form at S51 revealed a greater activation of this pathway, which attenuates translation initiation and protein synthesis and induces protein degradation in the brains of FD hamsters ($p < 0.05$). In addition, melatonin administration to FD hamsters was able to deactivate this pathway ($p < 0.05$) (Fig. 3).

High-Fructose Diet Impairs Degradative Systems

Given the possibility of the presence of unfolded or misfolded proteins in the brain of FD animals, we evaluated some cell quality control mechanisms responsible for eliminating these defective proteins, such as the ubiquitin-proteasome system and autophagy. The ubiquitin-proteasome system results revealed no significant differences in 20S proteasome activity nor in the amount of ubiquitinated proteins (Fig. 4). Chaperone-mediated autophagy (CMA) is another type of low capacity proteolytic degradation system. Protein expression of the specific marker lysosome-associated Membrane Protein Type 2a (LAMP-2A) indicated no significant differences in CMA between the four experimental groups (Fig. 5).

Macroautophagy is a high-capacity lysosomal degradation process that is activated as a consequence of high cellular stress, such as bioenergetic failure or accumulation of protein aggregates. To study the viability of this lysosomal system, we analyzed the protein expression of cathepsin D and found that it was significantly higher in FD animals ($p < 0.01$) (Fig. 6a). Expression of beclin-1, which is part of the complex inducer of vesicle nucleation, was higher in the brains of hamsters fed with high fructose than in ND animals ($p < 0.05$). Interestingly, melatonin administration to FD animals reduced the beclin-1 protein levels ($p < 0.01$) (Fig. 6b).

Microtubule-associated protein 1 light chain 3 (LC3) in its lipidated form (LC3-II) can serve as an autophagosomal

Table 1 Effect of diet and treatment with melatonin on body and intake parameters

	Normal diet		Fructose diet	
	Control	Melatonin	Control	Melatonin
Intake (g/day)	14.5 ± 0.05	13.3 ± 0.8	14.3 ± 0.05	14.3 ± 0.10
Weight increase (g)	63.1 ± 10.4	57 ± 14	50 ± 15	46.2 ± 30.6
Brain weight (g)	1.77 ± 0.05	1.75 ± 0.09	1.87 ± 0.06	1.86 ± 0.05

Data are expressed as the mean ± standard deviation

marker. Our results showed that the diet rich in fructose produced an increase in LC3-I but a decrease in LC3-II levels ($p < 0.05$) (Fig. 6d), demonstrating the presence of a smaller number of autophagosomes in the brain of FD hamsters. To determine the autophagic flux, we quantified the protein expression of sequestosome-1 (SQSTM1/p62), which binds structures, protein aggregates, or other toxic cellular waste and targets them for selective degradation by macroautophagy. We found an accumulation of p62 in the brain of DF hamsters ($p < 0.01$) (Fig. 6e), confirming lower autophagic flux. The LC3-II and p62 protein levels were not affected by the melatonin treatment.

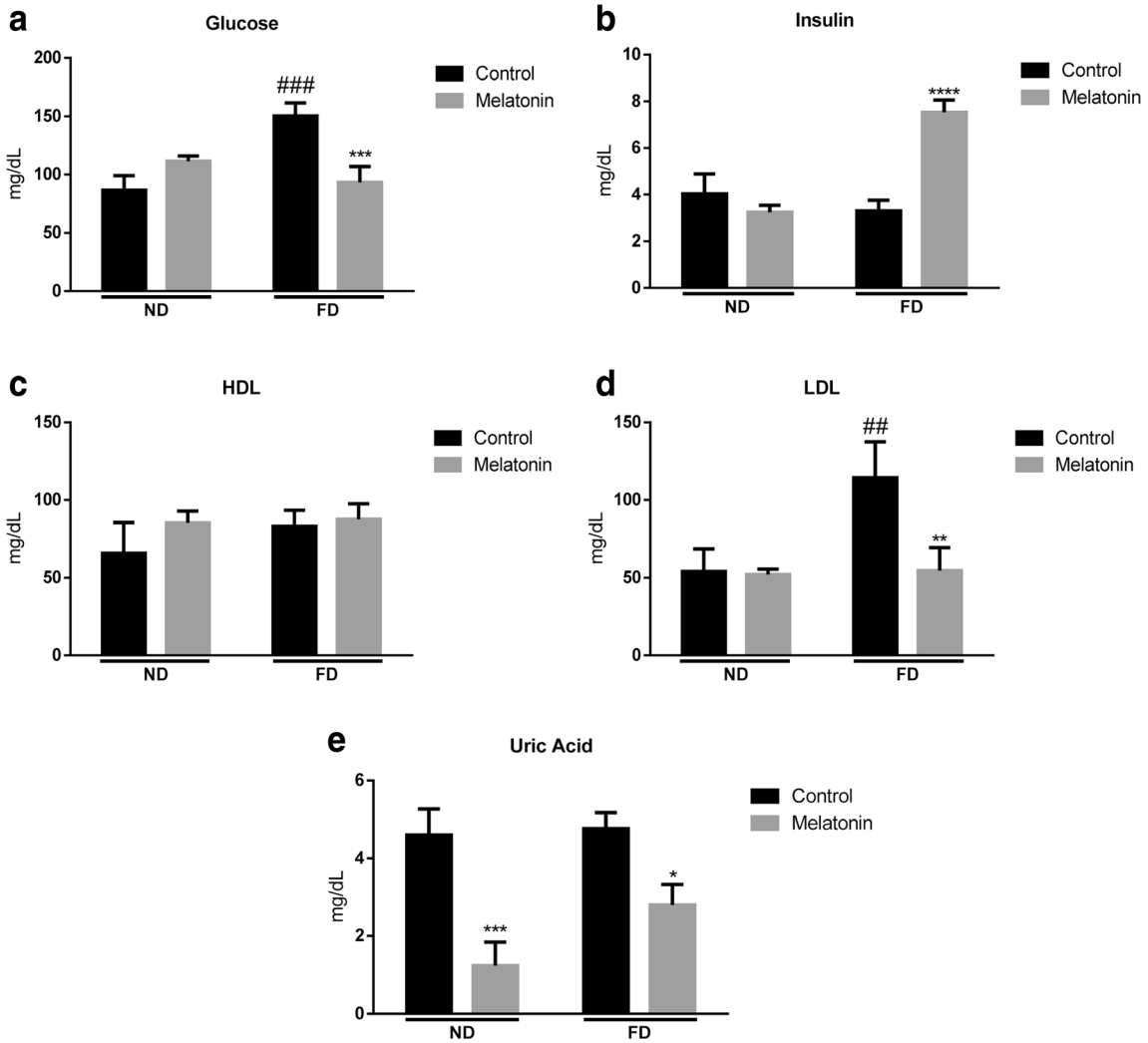


Fig. 1 Blood levels of glucose (**a**), insulin (**b**), high-density lipoprotein (HDL) (**c**), low-density lipoprotein (LDL) (**d**), and uric acid (AU) (**e**) in the four experimental groups: hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated (control) and treated with melatonin. The results are expressed in milligrams per deciliter of blood plasma. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

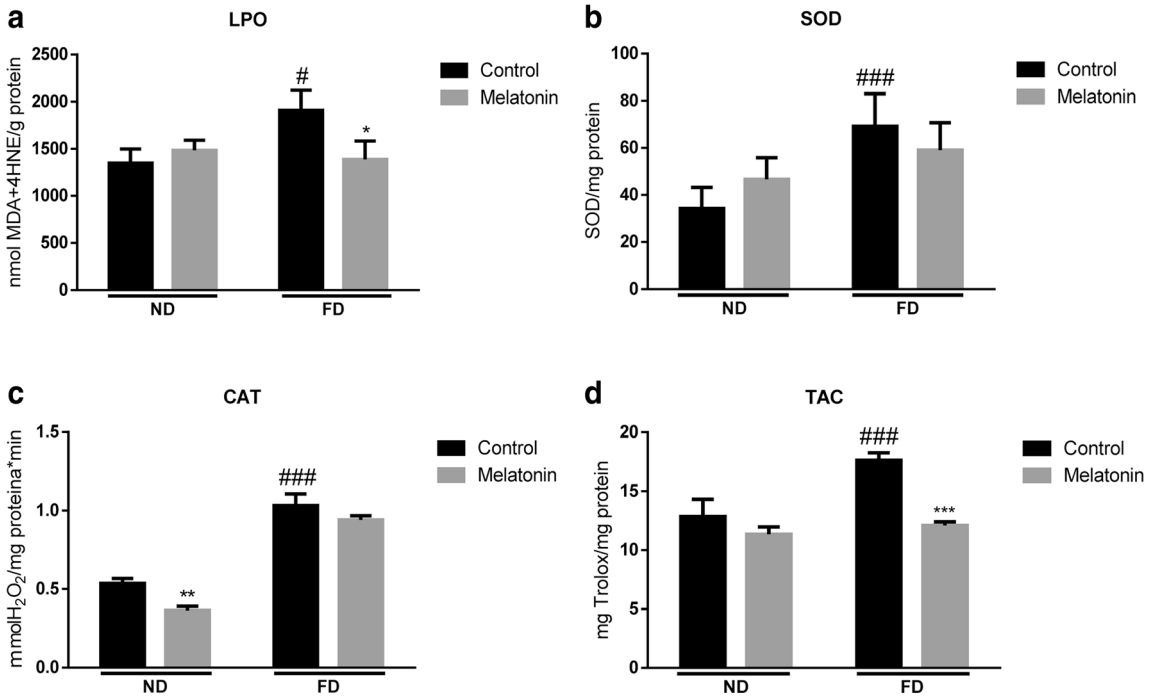


Fig. 2 Lipid peroxidation (LPO) (**a**), superoxide dismutase activity (SOD) (**b**), catalase activity (CAT) (**c**), and total antioxidant capacity (TAC) (**d**) in brains of the four experimental groups: hamsters fed a normal diet (ND), high-fructose diet (FD), untreated (control), and treated with melatonin. Data are expressed as the mean ± standard deviation. * vs. control; # vs. ND. The number of symbols marks the level of significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

High-Fructose Diet Induces the Accumulation of some Neurodegenerative Markers

A common feature of many neurodegenerative disorders, such as AD and PD, is the presence of potentially toxic protein aggregates. To evaluate whether diets with a high content of fructose induced the accumulation of these types of aggregates in the brain, we analyzed some markers of neurodegeneration, such as β-amyloid (1–42), α-synuclein, and tau phosphorylation, in our experimental model. Immunoblot analysis of β-amyloid peptide (1–42), which is a central component of neuritic (senile) plaques, revealed a higher content of this toxic peptide in the brains of FD hamsters than in the brains of ND animals ($p < 0.01$). In addition, melatonin was able to reduce β-amyloid (1–42) in FD hamsters, but the difference showed no statistical significance (Fig. 7a). Immunodetection of α-synuclein, which is usually present in the presynaptic terminals, in the nuclear envelope, and in cytoplasmic inclusion bodies, such as the Lewy bodies, showed no significant differences in α-synuclein content between the four experimental groups. Despite this result, we observed a trend towards a higher level of α-synuclein protein accumulation in the brain of FD animals that was mitigated by melatonin (Fig. 7b). Finally, we studied tau phosphorylation at the following residues: S199, T205, S396, and S404 whose deposits are pathological characteristics of several tauopathies. The levels of tau phosphorylated at S199 and S404 were significantly higher in the brain of FD rodents ($p < 0.01$) (Fig. 7c, f), and melatonin treatment was able to reduce tau phosphorylation at S404 ($p < 0.01$), but not at S199 (Fig. 7c). On the other hand, we observed that the protein levels of p-tau (T205) and p-tau (S396) in the brains from animals fed with FD were lower than those in ND animals ($p < 0.01$), remaining unaltered by melatonin treatment (Fig. 7d, e).

Discussion

Fructose is one of the most widely used sugars in the food industry, and it is used as a sweetener for processed foods. Among the consequences of high and continued consumption of fructose is the development of obesity and diabetes mellitus [1]. However, the CNS consequences from high-fructose intake are poorly discussed in the scientific literature. We focused this study on investigating the possible cellular alterations at the cerebral level that may be associated with high-fructose consumption. At the same time, we tested the effects of exogenous administration of melatonin against these potentially harmful effects because melatonin is known to be a potent antioxidant and a modulator of metabolic pathways.

Several studies have associated excessive fructose consumption with the occurrence of hyperuricemia, IR, dyslipidemia, hyperglycemia, and increased plasma concentrations of

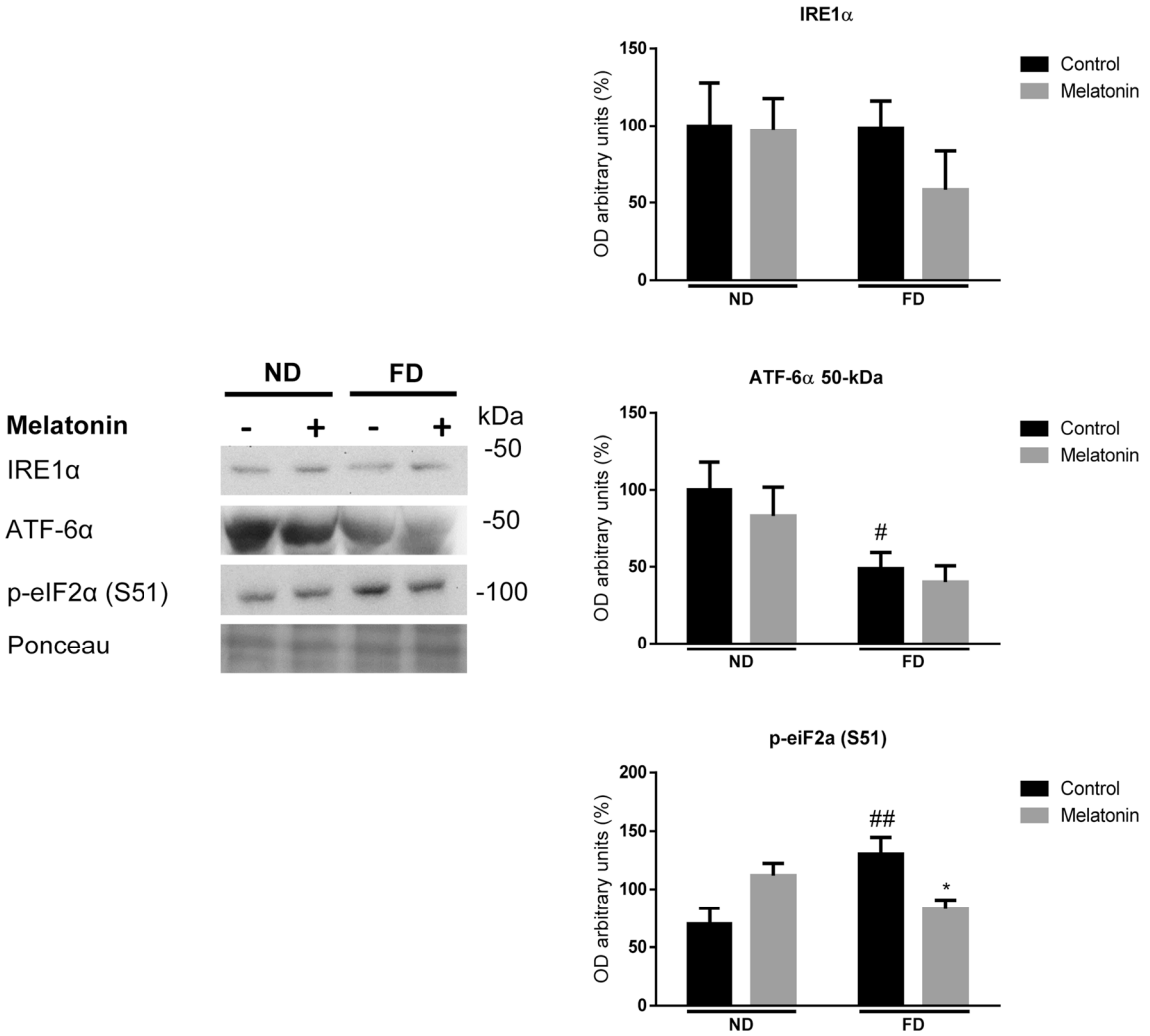


Fig. 3 Representative immunoblot for Ire1α, ATF-6α, and p-eIF2α in the brain of hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated and treated with melatonin. The histograms show the optical densities from three independent experiments. Data are expressed as the means ± standard deviation. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

triglycerides and LDL cholesterol together with a decreased concentration of HDL cholesterol [33–35]. To determine whether 10 weeks of exposure to a high-fructose diet could cause this type of systemic effects, we analyzed some of these blood parameters. Although no changes were observed in body weight and total daily food intake, probably due to the short-term exposure to this diet [36], the blood parameter results indicated the presence of a profile that was closely related to metabolic syndrome [37]. Fructose enhanced blood glucose levels without increasing insulin levels. Several studies seem to correlate this hyperglycemia with a predisposition to type II diabetes because fructose consumption may alter insulin secretion [38] by disrupting beta cell function in pancreatic islets due to an increase in hepatic diacylglycerol (DAG), a secondary messenger that is produced during the generation of triglycerides and that leads to IR by disrupting signal transduction from the insulin receptor [39]. In addition, this diet resulted in increased plasma LDL cholesterol, which has already been described in previous studies, and an increase in uric acid [40]. The high levels of LDL cholesterol may result from fructose intake-associated hyperlipidemia [33]. However, we did not observe hyperuricemia, which has been associated with the manifestation of cognitive deficits [41], probably due to the short duration of this high-fructose exposure. Despite these observations, some of the results obtained in blood could be related to the appearance of IR. In fact, several investigations have noted a strong correlation between the presence of IR and early onset of AD [6–9]. This relationship is regarded to lead to an initial change in low-grade neuroinflammation in AD. Thus, brain IR and AD have even been discussed in terms of type 3 diabetes [5, 42]. It has also been suggested that hyperglycemia can increase the synthesis of β-amyloid protein and lead to dysfunction in synaptic transmission [43].

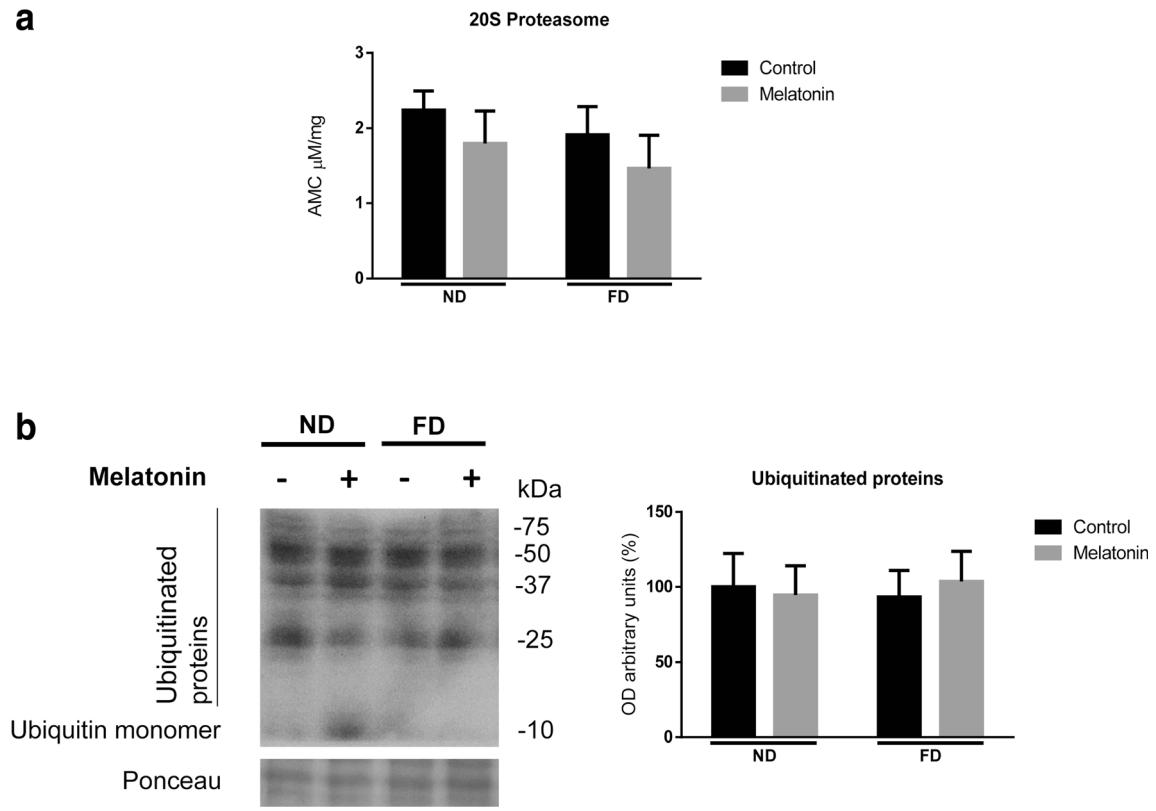


Fig. 4 20S proteasome activity (**a**) and ubiquitin detection by western blot (**b**) in the brain of hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated and treated with melatonin. The histogram shows the optical densities from three independent experiments. Data are expressed as the means ± standard deviation. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

Increased fructose consumption has recently been associated with high oxidative stress in the brain, linking this relationship with the pathogenesis of neurodegenerative diseases [34]. Our results seem to corroborate this association, confirming the presence of oxidative damage in the brain of FD animals. In addition, one of the predisposing factors for increased oxidative stress is the manifestation of hyperglycemia [44], as we observed in FD hamsters. Even so, an antioxidant response against the high-fructose diet-induced oxidative damage, including both enzymatic and probably non-enzymatic antioxidants, was trigged in the brain of FD rodents. In fact, the effect of melatonin treatment on redox parameters suggests that the antioxidant response induced by the high-fructose diet has a high component of non-enzymatic antioxidants, which may no longer be necessary in the presence of melatonin.

Increased oxidative stress can contribute to alterations of homeostatic control mechanisms. Moreover, there is a direct relationship between oxidative stress and ER stress [45], and ER stress-induced apoptosis is implicated in the occurrence of AD and PD because of the postmitotic nature of neurons,

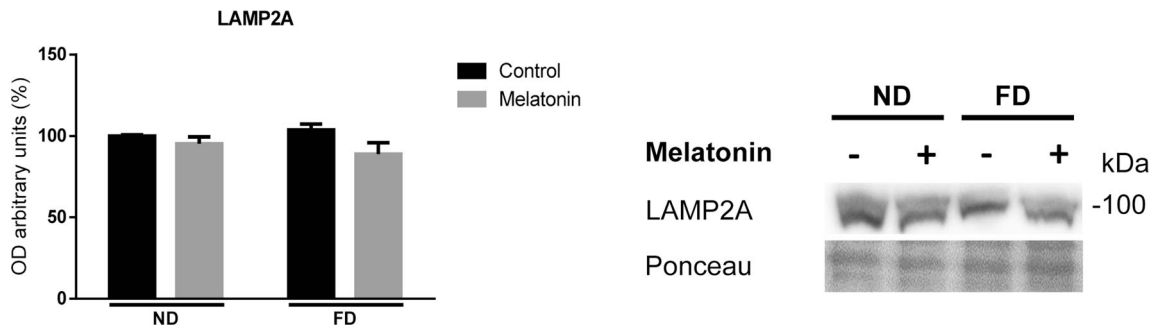


Fig. 5 Representative immunoblot for LAMP2A in the four experimental groups: hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated (control) and treated with melatonin. The histogram shows the optical densities from three independent experiments. Data are expressed as the means ± standard deviation. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

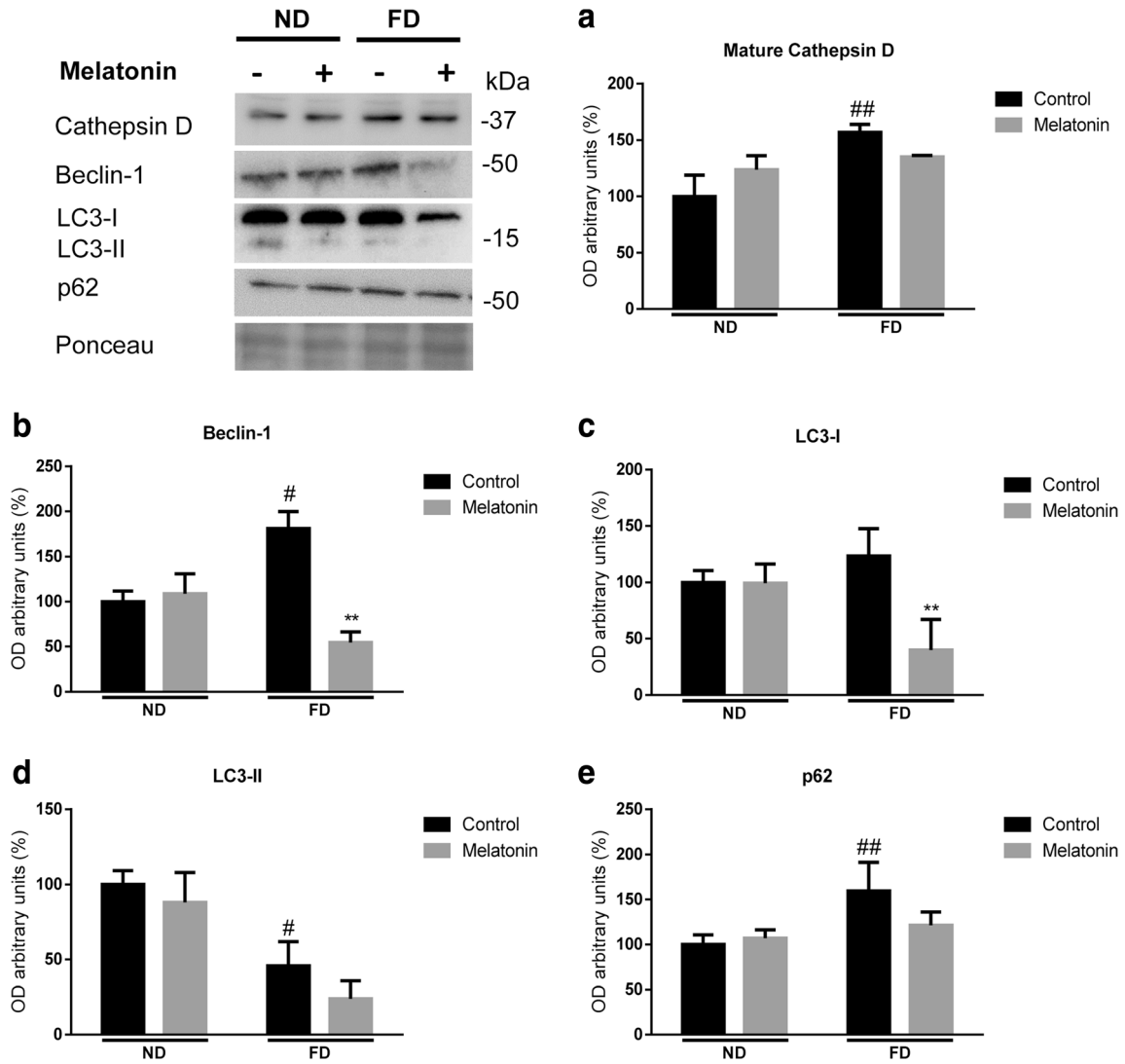
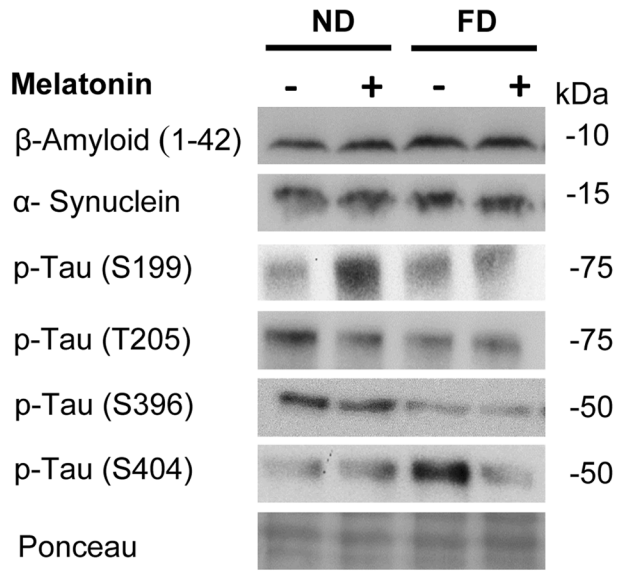
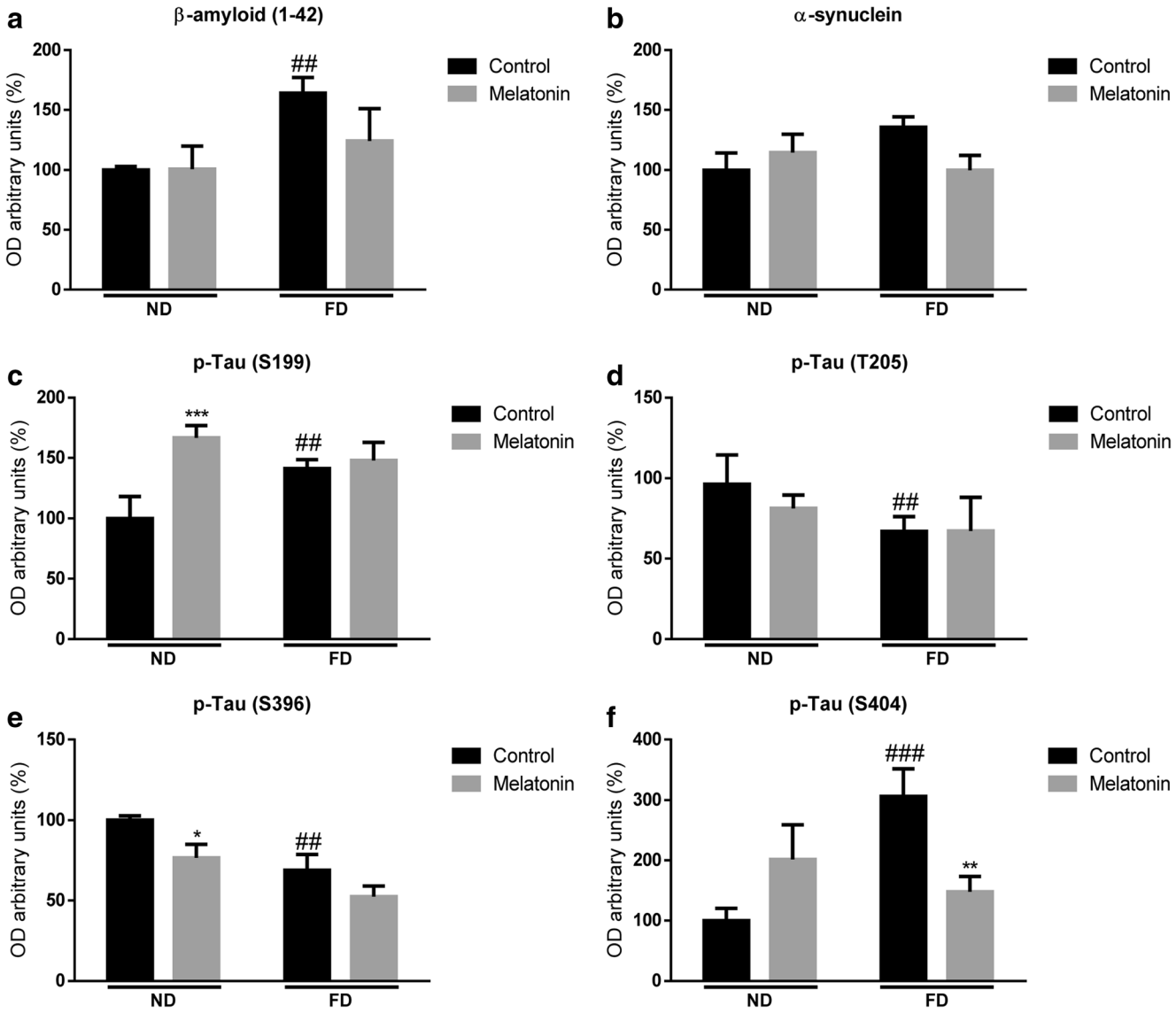


Fig. 6 Histograms showing the optical densities from three independent western blot experiments against cathepsin D (**a**), beclin-1 (**b**), LC3-I (**c**), LC3-II (**d**), and p62 (**e**) in the brain of hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated and treated with melatonin. Values are the means ± standard deviation. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

which makes them more susceptible to these types of events [46]. Several studies have addressed the relationship between fructose and ER stress in the liver, but this relationship has been poorly studied in the brain. These studies showed that high-fructose diets activate the eIF2α and IRE1 pathways of the UPR, which are related to hepatic steatosis and IR [47]. In the present study, we detected changes that seem to indicate that fructose produces alterations in protein folding in the ER lumen and a consequent adaptation to ER stress in the brain, which includes triggering of an UPR that is characterized by the deactivation of the ATF-6 pathway and activation of the eIF2α pathway.

Although when under ER stress, activation of the ATF-6α arm is not essential for the development and survival of neurons, and its deactivation in DF animals may be a contributor to misfolded protein accumulation, activation of ER stress-induced apoptosis, and the consequent onset of neurodegeneration. In fact, in animal models of PD, it was demonstrated that this pathway has a neuroprotective role against the loss of dopaminergic neurons [48, 49]. On the other hand, high-fructose intake resulted in eIF2α activation. The eIF2α pathway attenuates translation initiation and protein synthesis and induces protein degradation, suggesting that this type of adaptive responses is triggered against the accumulation of abnormal proteins in the brain of FD animals. In spite of this, the alterations in oxidative stress and ER stress induced in the brain by fructose appear to have no effects on the activation of mechanisms that degrade proteins on a molecule-by-molecule basis (proteasome and CMA), either because damaged proteins are not accumulated and these mechanisms are unnecessary or because they are overtaken, and then, other mechanisms with a greater capacity, such as macroautophagy,


ND
FD
Melatonin
- + - +
kDa
β-Amyloid (1-42)
-10
α- Synuclein
-15
p-Tau (S199)
-75
p-Tau (T205)
-75
p-Tau (S396)
-50
p-Tau (S404)
-50
Ponceau


a
β-amyloid (1-42)
b
α-synuclein
c
p-Tau (S199)
d
p-Tau (T205)
e
p-Tau (S396)
f
p-Tau (S404)
OD arbitrary units (%)
Control
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◀ **Fig. 7** Histograms showing the optical densities from three independent western blot experiments against β-amyloid (**a**), α-synuclein (**b**), p-tau (S199) (**c**), p-tau (T205) (**d**), p-tau (S396) (**e**), and p-tau (S404) (**f**) in the brains of hamsters fed a normal diet (ND) and high-fructose diet (FD), untreated and treated with melatonin. Data are expressed as the means ± standard deviation. Statistical comparisons: * vs. control; # vs. ND. The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$

are activated. It has been proposed that the accumulation of advanced glycosylation endproducts (AGEs)-modified proteins derived from high-fructose consumption or chronic hyperglycemia [50] activates eIF2α [51] and leads to the activation of mechanisms for the identification and removal of damaged proteins, such as the ubiquitin-proteasome system and autophagy-lysosomal system. Thus, the age-related impairment of the proteolytic efficiency may exacerbate protein aggregation diseases, such as AD [52], in individuals with a high-fructose intake. However, these proteolytic systems may also be affected by other age-independent situations. For example, it has been shown in the liver that free fatty acid-induced oxidative stress leads to proteasome dysfunction, which mediates obesity-induced ER stress and IR [53] and that, in our case, could increase accumulation of AGEs and led to the appearance of protein aggregates. In fact, we found a higher level of beclin-1 expression in FD animals, suggesting that fructose induces the accumulation of protein aggregates in the brain that cannot be degraded by unfolded monomer protein degradation systems and that induce the activation of high-capacity alternative mechanisms, such as macroautophagy. Despite this, we observed a lower expression of LC3-II, which may indicate decreased synthesis of autophagosomes (less autophagy induction) or increased fusion with lysosomes (greater autophagic flux).

Cells have to maintain an adequate lysosomal system to form autolysosomes and execute autophagy. The post-translational processing of cathepsin D to its mature form indicates a developed endosomal-lysosomal system. Since the brain of FD animals showed increased expression of mature cathepsin D, it can execute the last phases of the autophagic process. Furthermore, this upregulation of lysosomal cathepsins is probably a protective response to reduce the toxicity of diet-derived AGEs-modified proteins [54]. However, p62 accumulation demonstrated decreased autophagic flux, confirming that the decrease observed in LC3-II is due to decreased synthesis of autophagosomes. Taken together, these results indicate that FD induces an impairment of macroautophagy, which leads to the accumulation of p62-labeled aggregates in the brain. Although it has already been described that fructose supplementation alters the autophagic mechanism at various levels in the liver and white adipose tissue [55, 56], to our knowledge, this is the first report demonstrating this alteration in the brain.

It has been suggested that high-fructose diets may directly or indirectly increase the risk of neurodegeneration or cerebral dysfunction in animal models by increasing oxidative stress, which together with the proteolytic dysfunction favors the accumulation of abnormal proteins [57]. Despite the short time of exposure to the high-fructose diet, we observed an accumulation of beta-amyloid peptide (1–42), which is a hallmark of AD, because this isoform is less soluble than others [58]. In addition, even though this change was not statistically significant, the α-synuclein content was also higher in the brain of FD hamsters.

Tau protein is mainly found in CNS neurons and stabilizes microtubules, but when it is hyperphosphorylated, tau protein loses its effectiveness and starts to accumulate. In AD, this protein is abnormally phosphorylated at serines and threonines, such as at S396, S404, and T205 [59]. Although the brain of FD animals showed decreased expression of tau p-S396 and tau p-T205, tau phosphorylation at S404, a critical site for microtubule assembly, and at S199, which is involved in the formation of neurofibrillary tangles [60, 61], was increased in the brain of DF animals. These results suggest that excessive consumption of fructose could favor the occurrence of primary events of AD.

It has been suggested that melatonin may exert a beneficial role on the early stages of high-fructose-induced metabolic syndrome [62]. Our data support this hypothesis since administration of melatonin attenuated the blood metabolic changes caused by excessive fructose intake, increased insulin levels, and reestablished glucose and LDL levels. However, we must consider that the fundamental difference between nocturnally active rodents and diurnally active human leads to substantial differences between these organisms in relation to melatonin. While melatonin has generally been found to be antidiabetic in rodents, the opposite is true in human, at least at the levels of glucose tolerance and insulin secretion, which are reduced by melatonin even in normoglycemic health young adults [63–65]. These findings are underpinned by the effects of a gain of function mutation ("G allele") of the melatonin receptor gene MTNR1B, which is prodiabetic, after overexpression in pancreatic beta cells [66, 67]. Nevertheless, it seems possible that fructose toxicity in both humans and rodents may be similarly counteracted by melatonin in the human, not at the level of insulin secretion but by antagonizing the proinflammatory and prooxidant effects of fructose and their secondary consequences. In fact, in FD animals, melatonin treatment was able to reduce oxidative stress. However, melatonin administration to FD animals maintained the SOD and CAT levels, but reduced the TAC, suggesting that the antioxidant response induced by high-fructose diets includes the production of non-enzymatic antioxidants that are no longer necessary when melatonin is administered. In addition, melatonin deactivated the eIF2α arm of the UPR. Thus, its beneficial actions seem to primarily avoid the accumulation of abnormal proteins. Therefore, cells do not need to activate beclin1-mediated autophagy. Although melatonin treatment did not improve either

autophagosome formation or reduce the p62 levels, we observed a slight reduction in the β-amyloid (1–42), α-synuclein, and tau-p-S404 levels, supporting the neuroprotective role of melatonin against these accumulations [68, 69].

This study provides new relevant information on the early effects of high-fructose diets on the brain that, in the long term, will lead to a possible neuropathological manifestation. We found that early events of excessive fructose intake produced similar symptoms to type II diabetes and, in the brain, alterations in the redox system and in the mechanisms of detection and degradation of abnormal proteins, giving rise to early neurodegenerative alterations. Our data also showed the beneficial effect of melatonin on primary neurodegeneration events caused by excessive fructose consumption.

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Compliance with Ethical Standards

The Oviedo University Local Animal Care and Use Committee approved the experimental protocol. All experiments were carried out according to the Spanish Government Guide and the European Community Guide for Animal Care.

Conflict of Interest The authors declare that they have no conflicts of interest.

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