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Melatonin prevents the harmful effects of obesity on the brain, including at the behavioral level

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Abstract:	Obesity is a health problem caused by a diet rich in energy and the sedentary lifestyle of modern societies. A leptin deficiency is one of the worst causes of obesity, since it results in morbid obesity, a chronic disease without a cure. Leptin is an adipokine secreted in a manner dependent on the circadian rhythm that ultimately reduces food intake. We studied cellular alterations in brain of leptin-deficient obese animals and tested whether these alterations are reflected in abnormal behaviors. Obesity induced increases in oxidative stress and the unfolded protein response caused by endoplasmic reticulum stress. However, the subsequent signaling cascade was disrupted, blocking possible systemic improvements and increasing the production of misfolded proteins, that trigger autophagy. Up-regulated autophagy was not indefinitely maintained and misfolded proteins accumulated in obese animals, which led to aggresome formation. Finally, neurodegenerative markers together with anxiety and stress-induced behaviors were observed in leptin-deficient mice. As oxidative stress has an essential role in the development of these harmful effects of obesity, melatonin, a powerful antioxidant, might counteract these effects on the brain. Following treatment with melatonin, the animals' antioxidant defenses were improved and misfolded protein, proteasome activity and autophagy decreased. Aggresome formation was reduced due to the reduction in the levels of misfolded proteins and the reduction in tubulin expression, a key element in aggresome development. The levels of neurodegenerative markers were reduced and the behaviors recovered. The data support the use of melatonin in therapeutic interventions to reduce brain damage induced by leptin deficiency-dependent obesity.

MELATONIN PREVENTS THE HARMFUL EFFECTS OF OBESITY ON THE BRAIN, INCLUDING AT THE BEHAVIORAL LEVEL

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Abstract

Obesity is a health problem caused by a diet rich in energy and the sedentary lifestyle of modern societies. A leptin deficiency is one of the worst causes of obesity, since it results in morbid obesity, a chronic disease without a cure. Leptin is an adipokine secreted in a manner dependent on the circadian rhythm that ultimately reduces food intake. We studied cellular alterations in brain of leptin-deficient obese animals and tested whether these alterations are reflected in abnormal behaviors. Obesity induced increases in oxidative stress and the unfolded protein response caused by endoplasmic reticulum stress. However, the subsequent signaling cascade was disrupted, blocking possible systemic improvements and increasing the production of misfolded proteins, that trigger autophagy. Up-regulated autophagy was not indefinitely maintained and misfolded proteins accumulated in obese animals, which led to aggresome formation. Finally, neurodegenerative markers together with anxiety and stress-induced behaviors were observed in leptin-deficient mice. As oxidative stress has an essential role in the development of these harmful effects of obesity, melatonin, a powerful antioxidant, might counteract these effects on the brain. Following treatment with melatonin, the animals' antioxidant defenses were improved and misfolded protein, proteasome activity and autophagy decreased. Aggresome formation was reduced due to the reduction in the levels of misfolded proteins and the reduction in tubulin expression, a key element in aggresome development. The levels of neurodegenerative markers were reduced and the behaviors recovered. The data support the use of melatonin in therapeutic interventions to reduce brain damage induced by leptin deficiency-dependent obesity.

Introduction

The prevalence of the overweight and obesity epidemics have significantly increased in the last three decades, including a dramatic rise in the prevalence of both disorders among adolescents. Currently, 39% of the global population is overweight and 13% is obese [1]. Overweight and obesity are the fifth leading causes of death worldwide; therefore, obesity and its associated health problems urgently require new strategies for effective treatments.

Obesity is a risk factor for many diseases such as diabetes, musculoskeletal disorders, some cancers, renal pathologies and, primarily cardiovascular diseases [2]. In addition, central nervous system damage induced by obesity has recently been reported [3,4]. According to animal studies, high-energy diet damages the structure and function of the hippocampus [5]. The brain is particularly susceptible to oxidative stress due to its high glucose and oxygen consumption [6], which generates a large amount of reactive oxygen species (ROS), and its poor antioxidant defense system [7].

Oxidative stress damages macromolecules and ultimately leads to dysfunctional neurons. Thus, cells (particularly postmitotic cells such as neurons) contain quality control mechanisms such as the unfolded protein response (UPR), proteasome and autophagy to remove damaged proteins [8]. When unfolded/misfolded proteins accumulate in the endoplasmic reticulum (ER), ER stress occurs and induces the UPR in order to decrease protein synthesis and increase the synthesis of chaperones and the activity of the ubiquitin/proteasome system [9,10]. If this response is not able to repair or avoid the accumulation of unfolded/misfolded proteins, the proteasome [11] and autophagy, particularly chaperone-mediated autophagy (CMA) and macroautophagy [12], are responsible for removing these proteins. Unfortunately, if these mechanisms fail, aggresomes begin to accumulate, hampering vesicle traffic. As shown in a recent publication by our group, autophagy is altered in the livers of obese individuals [13].

Obesity is caused by several factors, physiological to psychological conditions, and some unknown factors. However, leptin is one of the main factors, as a reduction in its levels or receptor inefficiency leads to morbid obesity [14,15].

Leptin, a 16 kDa adipokine, is an anorexigenic peptide hormone synthesized in and secreted from peripheral adipocytes. Upon binding to its receptor within the hypothalamus, leptin induces a biochemical cascade that ultimately reduces food intake and increases energy expenditure. This peptide is secreted with circadian rhythmicity, with maximal production in the middle of the night [16]; thus, leptin is inevitably associated with melatonin.

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland and plays a fundamental role in the control of circadian rhythms [17]. In fact, its relation with leptin has been established. According to several studies, melatonin administration decreases plasma and serum leptin levels in rats [18-20]. Moreover, pinealectomy results in an increase in circulating leptin concentrations, whereas the circadian rhythm of leptin secretion remains unchanged [21]. In summary, melatonin administration reduces serum leptin concentrations in both pinealectomized and normal rats.

Melatonin has many other functions in addition to its Zeitgeber role. Melatonin is one of the best known natural antioxidants [22,23]. The high efficiency of melatonin as an antioxidant depends on its ability to easily cross cell membranes due to its amphipathic nature [12]. Melatonin is also implicated in changes in body weight, depending on the photoperiod [24]. This molecule promotes weight loss by stimulating non-shivering thermogenesis and the recruitment of brown fat in small mammals [25]. In addition, melatonin regulates many other aspects of body weight and adiposity, such as energy intake and expenditure, glucose uptake [26], the serum lipid profile, blood pressure and inflammation [27]. Numerous publications have confirmed the regulatory roles of melatonin in the cell cycle, apoptosis and autophagy [12,17,28]. These effects of melatonin have been poorly studied in relation to its influence on actions of leptin in the brain.

Based on these considerations, the present study thoroughly analyzes the neuronal and behavioral alterations in leptin-deficient mice and whether a melatonin treatment counteracts the negative effects. Leptin deficiency-induced obesity caused diverse forms of cellular damage and led to abnormal behaviors through a similar pathway to aging, which was partially prevented by the melatonin treatment.

Materials and methods

Animals

Sixteen six-week-old male wild-type mice (C57BL/6J) and sixteen six-week-old male leptin-deficient obese B6.V-Lepob/J (ob/ob) mice were purchased from Charles River Laboratories (Barcelona, Spain). The ob/ob mice are characterized by hyperphagia, massive obesity (these mice weigh up to four times more than wild-type mice), transient hyperglycemia, and elevated plasma insulin concentrations (10-50 times higher than normal) [29]. This syndrome is inherited as a single autosomal recessive gene located on chromosome 6 [29]. The mice were housed two per cage under a 12:12 hour dark:light cycle at 22 ± 2 °C. The animals received tap water and a standard chow diet *ad libitum*. Higher food intake and the subsequent increase in the body mass index of the ob/ob mice compared with wild-type animals were reported previously [13].

Body and tissue parameters

All of the animals were weighed at the beginning (baseline) and end of the experiment (sacrifice) and both brain and body weights were recorded.

Melatonin treatment

After a two-week acclimatization period, animals were randomly divided into four groups with eight mice per group: the untreated control groups of wild-type and ob/ob mice (WC and ObC, respectively) and the melatonin-treated groups of wild-type and ob/ob mice (WM and ObM, respectively).

Two hours after the lights were switched off, melatonin (Sigma-Aldrich, St. Louis, MO, USA) was intraperitoneally injected at a dose of 500 μg/kg body weight daily for 4 weeks. Melatonin was dissolved in 0.5% absolute ethanol:saline. Animals in the control groups received a comparable dose of vehicle via the same route for the same treatment duration.

The animals were euthanized by decapitation, and the brain of each mouse was immediately removed, flash-frozen in liquid nitrogen and stored at -80 °C until further analysis. The experimental protocol was approved by the Oviedo University Animal Care and Use Committee. All experiments were performed

according to the Spanish Government Guide and the European Community Guide for Animal Care (Council Directive 86/609/EEC).

Tissue processing

The brain of each mouse was homogenized in buffer (pH 7.5) containing 50 mM sodium phosphate buffer, 100 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF and 1% Triton at a ratio 1:10 (w:v) using an Ultra-Turrax T25 Mixer (IKA, Staufen, Germany). The homogenates were centrifuged at 900 g for 6 minutes at 4 °C. Supernatants containing proteins were collected, aliquoted and frozen at -80 °C until further analysis.

Protein quantification

The Bradford method was used to quantify the protein concentrations in the brain homogenates [30].

Oxidative damage

Protein oxidative damage

Protein oxidative damage was determined by measuring the concentrations of carbonylated proteins using the methods described by Levine *et al.* [31], with the modifications reported by Coto-Montes and Hardeland [32]. The results are presented as nmol carbonylated protein/mg protein.

Antioxidant enzyme activities

Total antioxidant activity

Total antioxidant activity (TAA) was determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation radical method [33,34]. The results are expressed as mg equivalents Trolox/mg protein.

Superoxide dismutase activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using the method reported by Martin *et al.* [35]. This enzyme inhibits hematoxylin auto-oxidation to the colored compound hematein. The results are expressed as SOD units/mg protein.

Catalase activity

Catalase (CAT; EC 1.11.1.6) activity was assayed using the method reported by Lubinsky and Bewley [36] and H_2O_2 as the substrate. The results are expressed as μmol H_2O_2/mg protein*minute.

Glutathione peroxidase activity

Glutathione peroxidase (GPx; EC 1.11.1.9) catalyzes the reduction of H_2O_2 to H_2O using the reducing agent reduced glutathione (GSH), which is oxidized to glutathione (GSSG). The assay was performed by monitoring the oxidation of NADPH [37]. The results are expressed as μmol NADPH/mg protein*minute.

Glutathione reductase activity

Glutathione reductase (GR; EC 1.6.4.2) activity was measured using the method described by Goldberg and Spooner [38]. This enzyme catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH + H^+ as the substrate. The assay was performed by monitoring the oxidation of NADPH [37]. The results are expressed as μmol NADPH/mg protein*minute.

Enzyme-linked immunosorbent assay (ELISA)

Tumor necrosis factor α (TNF-α)

Brain levels of TNF-α were determined using a commercially available ELISA kit (KMC3011, Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The assay was performed according to the manufacturer's instructions, and the results are expressed as pg TNF-α/mg protein.

Interleukin 6 (IL-6)

Brain levels of IL-6 were determined using a commercially available ELISA kit (KMC0061, Novex, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The assay was performed according to the manufacturer's instructions. The results are expressed as pg IL-6/mg 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 μM AMC/mg protein.

Western blot immunoassay

Tissue homogenates (50 μg of protein per sample) were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and denatured by boiling at 100°C for 5 minutes. The samples were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V and subsequently transferred onto a polyvinylidene fluoride (PVDF) membranes (Immobilon TM-P; Millipore Corp., Bedford, MA, USA) at 350 mA.

The membranes were blocked with 5 or 10% (w/v) nonfat dry milk dissolved in Tris-buffered saline (TBS) (50 mm Tris-HCl, pH 7.5, 150 mm NaCl) for 1 hour at room temperature. Subsequently, the membranes were incubated with the following primary antibodies overnight at 4°C: anti-IRE1α (3294, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated-eIF2α (3398, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ATF-6α (sc-22799, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ubiquitin (3933, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-LAMP2A (ab18528, Abcam, Cambridge, UK), anti-Beclin-1 (sc-10086, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LC3 (PD014, Medical & Biological Laboratories CO., LTD., Naka-ku Nagoya, Japan), anti-p62 (H00008878-M01, Abnova, Walnut, CA, USA), anti-β-actin (AC-15, Sigma-Aldrich, St.

Louis, MO, USA), anti-α-tubulin (sc-23948, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-vimentin (5741, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-amyloid (2454, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-α-synuclein (2642, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Tau p-S^{199}, anti-Tau p-T^{205}, anti-Tau p-S^{396} and anti-Tau p-S^{404} (44779G, Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).The antibodies were diluted in TBS buffer containing 1% (w/v) nonfat dry milk and 0.02% sodium azide. After three 10 minutes washes in Tris-buffered saline containing Tween (TBS-T) (50 mm Tris/HCl, pH 7.5, 150 mm NaCl, and 0.05% Tween-20), the membranes where incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (anti-goat (A4174, Sigma-Aldrich, St. Louis, MO, USA), anti-mouse (7076, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-anti-rabbit (7074, Cell Signaling Technology, Inc., Danvers, MA, USA)) diluted in TBS buffer containing 1% (w/v) nonfat dry milk for 1 hour at room temperature, followed by three 10 minutes washes in TBS-T.

The membranes were developed using a chemiluminescent substrate (WBKLS0500, Merck Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Protein levels were quantitated using Image Studio Lite 5.2.5 software (LI-COR Biotechnology, Lincoln, NE, USA). Variations in the levels of the typical housekeeping proteins (GAPDH, β-actin and α-tubulin) were found, so Ponceau S staining was used to ensure equal loading [39]. The results were expressed as percent change from the WC sample. Due to high LC3-I protein expression and low LC3-II protein expression, LC3-I signal overexposures covered the low LC3-II signal. Then, we have separately pictures of each protein, since the problem was always that both proteins needed different exposure times to get the best ones, again because of low LC3-II protein expression.

Protein identification by peptide mass fingerprinting

Brain protein homogenates (15 μg) were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and boiled at 100°C for 5 minutes to completely denature the proteins. Both samples and prestained molecular weight standards (Precision Plus Protein All Blue Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA)) were loaded onto 10% SDS-PAGE gels. Gels were electrophoresed at 100 V, stained with a mixture of 30% (v:v) methanol, 10% (v:v) acetic acid and

0.01% (w:v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and destained using a mixture of 40% (v:v) methanol and 10% (v:v) acetic acid. Images of the stained gels were captured using a GS-800 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and semiquantitatively analyzed using Image Studio Lite 5.2.5 software (LI-COR Biotechnology, Lincoln, NE, USA). The results were expressed as percent change from the WC sample.

The bands of interest were processed according to the protocol described by Oliván *et al.* [40]. Bands of interest were sent to the proteomics laboratory of Inbiotec S.L. (León, Spain) for identification, where the proteins were digested according to the method reported by Havlis *et al.* [41] and processed for further analysis using the method reported by Jami *et al.* [42].

Protein candidates produced by this combined peptide mass fingerprinting/tandem MS search were considered valid when the global Mascot score was greater than 85 with a significance level of $p<0.05$.

Porsolt Swim Test (PST)

The PST developed by Porsolt in 1977 [43] is a model of behavioral hopelessness based on the induction of immobility in animals in response to a stressful stimulus, and the results are interpreted as the animals' susceptibility to negative moods.

The behavioral test was conducted in a designated behavior room to provide a suitably quiet environment and the necessary equipment for behavioral assays. The animals were placed in a transparent glass cylinder (13 cm diameter x 24 cm high), filled with 22 ± 2 °C water. The animals were subjected to a single-phase test for 4 minutes. Their behaviors were recorded and then designated as either immobility or mobility. We have considered mobility as vigorous movements with the front and/or back legs in and out of the water, as proposed by Gersner *et al.* [44]. Passive displacement was considered as immobility. After each test, the mice were dried with a thermal blanket before being returned to their accommodation cage.

Statistical analysis

All results are presented as the mean values ± standard deviations (SD) of the means. All data presented representative data obtained from at least three separate experiments. The results were analyzed using two-way analysis of variance (ANOVA), with the first effect analyzing the phenotype (WC and ObC) and the second effect analyzing the treatment (WC and ObC or WM and ObM), and differences between individual means were analyzed with the Bonferroni post hoc test. The differences were considered statistically significant when $p<0.05$. Statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Body and tissue parameters

As shown in Table 1, body weight at sacrifice was higher in ob/ob mice than in wild-type mice ($p<0.001$); however, the administration of melatonin did not produce changes in this parameter in either mouse strain. Interestingly, brain weight was significantly lower in ob/ob than in wild-type mice ($p<0.01$), and the melatonin treatment slightly increased the brain weight in ob/ob animals to levels similar to the wild-type mice, although the difference was not significant (Table 1).

Leptin-deficient mice exhibit greater oxidative damage in the brain

Oxidative damage to proteins was evaluated by measuring the protein carbonyl content and the results showed greater oxidative damage in brains from leptin-deficient (ob/ob) mice than in brains from wild-type animals ($p<0.05$). The melatonin treatment did not alter oxidative protein damage in wild-type animals, but decreased protein carbonylation in ob/ob mice ($p<0.001$) (Fig. 1a). We next measured the levels of the main markers of the first line antioxidant defense system to elucidate the precise mechanism by which melatonin protected leptin-deficient mice.

The ABTS cation radical assay showed similar antioxidant capacities in the brains from both types of mice. We only detected significant changes after the melatonin treatment, which was able to reduce TAA levels in both strains ($p<0.001$ for WC compared with WM and $p<0.01$ for ObC compared with ObM), probably due to its well-described antioxidant properties (Fig. 1b). We measured the activities

of the main antioxidant enzymes to complete the analysis. SOD and CAT activities were also similar in both types of mice. Melatonin slightly decreased SOD activity, whereas it significantly increased CAT activity in the brains from wild-type animals ($p<0.01$). Similarly, melatonin significantly decreased SOD activity and increased CAT activity in leptin-deficient mice ($p<0.05$ for SOD and $p<0.001$ for CAT) (Fig. 1c and d). GPx and GR activities also exhibited a similar pattern. Brains from ob/ob mice showed increased activities of both antioxidant enzymes compared with brains from wild-type animals ($p<0.05$ for GPx and $p<0.01$ for GR). Although the melatonin treatment induced a slight increase in GPx activity in wild-type animals, this increase was only significant in ob/ob mice ($p<0.01$) (Fig. 1e). In contrast, the melatonin treatment was only able to significantly increase GR activity in the wild-type mice ($p<0.01$) (Fig. 1f). In summary, the melatonin treatment induced a readjustment in the antioxidant system, improving the redox balance, particularly in ob/ob animals, by stimulating SOD, CAT and GPx activities.

Leptin deficiency-induced obesity alters brain cytokine levels

We measured TNF-α and IL-6 levels to evaluate whether a leptin deficiency alters inflammation status in the brain. TNF-α levels were lower in ob/ob mice ($p<0.001$) and IL-6 levels were higher in leptin-deficient ob/ob mice than in wild-type mice ($p<0.001$). The melatonin treatment also exerted different effects on both strains by reducing TNF-α levels and increasing IL-6 levels in wild-type animals ($p<0.05$ for TNF-α and $p<0.001$ for IL-6) and increasing the levels of both cytokines in ob/ob mice ($p<0.01$ for TNF-α and $p<0.001$ for IL-6) (Fig. 2).

The leptin deficiency increases ER stress

Oxidative stress, inflammation and ER stress are concatenated processes that are usually activated in cells in response to stress conditions [45]. Under many pathophysiological conditions, the ER stress response termed the UPR attempts to alleviate protein misfolding and restore an efficient protein-folding environment. The three signaling pathways of the UPR, inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase (PKR)–like endoplasmic reticulum kinase (PERK), and

activating transcription factor-6α (ATF-6α), were assessed to evaluate whether the UPR is activated in the leptin-deficient mice [46,47]. Thus, the levels of IRE1α, p-eIF2α (phosphorylated-eukaryotic initiation factor 2α) and ATF-6α were measured. The levels of the IRE1α protein were similar in both strains of mice. Although the levels of p-eIF2α were reduced in ob/ob mice ($p<0.001$), the levels of ATF-6α pathway were increased ($p<0.01$) in these obese individuals. Wild-type mice treated with melatonin only exhibited an increase in the IRE1α protein content ($p<0.01$), which promotes cell survival by optimizing the functions of the ER and the cell. In contrast, melatonin reduced the expression of IRE1α and ATF-6α in ob/ob mice ($p<0.001$ for IRE1α and $p<0.01$ for ATF-6α) by decreasing cellular stress (Fig. 3a). The last cytoprotective branch of the UPR induces the expression of ER chaperones. Heat shock cognate 71 kDa (hsc70) protein belongs to the hsp70 family, whose main function is to prevent protein misfolding and aggregation [48]. The ob/ob mice exhibited decreased expression of hsc70 protein compared with wild-type animals ($p<0.001$), whereas the melatonin treatment decreased its expression in both strains of mice ($p<0.001$ for WC compared with WM and $p<0.001$ for ObC compared with ObM) (Fig. 3b).

Based on our results, the accumulation of unfolded/misfolded proteins in ob/ob mice triggers the UPR, which is characterized by the activation of ATF-6α. This increase in the ATF-6α levels seems to be required to facilitate tolerance to chronic stress [49]. The observed decrease in the IRE1α, ATF-6α and hsc70 levels induced by melatonin administration suggests an improvement in protein synthesis and folding.

Leptin-deficient mice exhibit reduced proteasome activity

As brains from ob/ob mice exhibited an accumulation of unfolded/misfolded and oxidatively damaged proteins, the cells may exhibit both a failure of the protein synthesis machinery or alterations in the degradation systems. These proteins should be eliminated by different protein quality control mechanisms, such as the ubiquitin-proteasome system. Leptin-deficient ob/ob animals showed a lower level of 20S proteasome activity than wild-type mice ($p<0.001$) (Fig. 4a) and a subsequent accumulation of ubiquitinated proteins ($p<0.001$) (Fig. 4b). Proteasome activity was also reduced by the melatonin treatment in both strains ($p<0.001$) (Fig. 4a), but did not alter the amounts of ubiquitinated proteins,

compared with the respective untreated group, which may be related to an improvement in protein synthesis induced by this indolamine (Fig. 4b).

Leptin-deficient ob/ob mice activate autophagy

The accumulation of altered proteins together with the impairment of the proteasome-ubiquitin system probably requires the activation of other protein quality control mechanisms to maintain protein homeostasis in the brains from obese mice. Thus, we studied the autophagic pathways by measuring the levels of lysosome-associated membrane protein type 2A (LAMP2A), a marker for CMA, and Beclin-1, microtubule-associated protein 1 light chain 3 (LC3) and the p62 protein, markers of macroautophagy. Leptin-deficient mice showed increased LAMP2A expression compared with wild-type animals ($p<0.01$) and the melatonin treatment reduced its expression ($p<0.01$) to the levels detected in wild-type animal (Fig. 5). Although ob/ob mice present impaired proteasome activity, CMA is triggered in an attempt to remove the damaged and unfolded/misfolded proteins.

Beclin-1, which plays an essential role in initiating autophagy [50], was expressed at higher levels in immunoblots of the brains from ob/ob mice than in the brains from wild-type animals ($p<0.001$) (Fig. 5). The most frequently used autophagy marker is LC3, whose lipidated form, known as LC3-II, is attached to the autophagosome membrane. Consistent with Beclin-1 results, the LC3-I and LC3-II proteins were expressed at higher levels in ob/ob mice than in wild-type animals ($p<0.05$ for LC3-I and $p<0.01$ for LC3-II), indicating that autophagosomes were formed in this leptin-deficient strain (Fig. 5). In addition, the melatonin treatment decreased the levels of all these autophagic biomarkers ($p<0.001$ for Beclin-1, $p<0.01$ for LC3-I and $p<0.001$ for LC3-II), suggesting that melatonin prevents the causes that trigger the Beclin-1-dependent autophagy induced by the leptin deficiency in ob/ob mice (Fig. 5).

The p62 protein interacts with LC3 to remove protein aggregates and is considered a marker of autophagic flux. The expression of the p62 protein was significantly increased in the ObC group ($p<0.05$), indicating that autophagic degradation was inefficient. This blockade seems to be resolved in the ObM group, as p62 expression was reduced to control levels ($p<0.001$). However, the levels of the p62 protein were significantly increased in the WM group ($p<0.01$), which may be related to antioxidant defense support [51,52] and were further increased by melatonin (Fig. 5).

Thus, leptin deficiency-induced obesity triggers both CMA and macroautophagy as compensatory cellular mechanisms for maintaining protein homeostasis.

The leptin deficiency induces aggresome formation

Cytoskeletal alterations are present in numerous diseases. Disruption of the cytoskeleton allows misfolded proteins to associate with cytoskeletal components and triggers the formation of inclusion bodies that are generically termed aggresomes [53]. Actin, microtubules, ubiquitinated proteins and vimentin, the intermediate filament that surrounds these elements, are main elements of these aggregates [54].

Immunoblots for β-actin and vimentin showed similar patterns, but α-tubulin expression differed from the levels of these proteins. Brain extracts from leptin-deficient obese mice showed increased expression of β-actin, α-tubulin and vimentin compared with wild-type animals ($p<0.001$). However, the melatonin treatment increased the β-actin ($p<0.001$) and vimentin ($p<0.05$) levels and significantly decreased α-tubulin expression in ob/ob mice ($p<0.001$) (Fig. 6). Disruption of microtubules blocks aggresome formation [55]; consequently, the significant decrease in the α-tubulin levels seems to support reduced aggresome formation in this experimental group.

The results observed in the ObC mice indicated effects on neuronal physiology and vesicle trafficking and could lead to the expression of neurodegenerative markers.

The leptin deficiency increases the accumulation of neurodegeneration markers

Based on the our results suggesting that some protein quality control mechanisms were impaired and cytoskeletal rearrangements occurred in ob/ob mice and findings that abnormal aggregates of cytoskeletal proteins are neuropathological signatures of many neurodegenerative diseases [56], we studied the expression of the main neurodegeneration markers in our experimental model of morbid obesity.

The β-amyloid and α-synuclein proteins are markers of Alzheimer's disease (AD) and Parkinson's disease (PD), respectively, and their deposition is also detected during age-related neurodegeneration.

Although ob/ob and wild-type mice presented equal amounts of the β-amyloid protein, with an unexpected, significant increase in wild-type animals treated with melatonin, ob/ob animals exhibited a significant increase in α-synuclein expression ($p<0.05$) (Fig. 7a).

Abnormal hyperphosphorylation of the microtubule-associated protein Tau is frequently observed in neurodegenerative diseases such as AD and even in normal age-related neurodegeneration. We analyzed four Tau phosphorylation sites: Tau p-S^{199}, Tau p-T^{205}, Tau p-S^{396} and Tau p-S^{404}. The levels of phosphorylation at these sites were similar but were significantly higher in brains from leptin-deficient ob/ob mice ($p<0.05$ for Tau p-S^{199}, $p<0.001$ for Tau p-T^{205}, $p<0.01$ for Tau p-S^{396} and $p<0.001$ for Tau p-S^{404}) (Fig. 7b). Although the melatonin treatment did not alter the β-amyloid content in ob/ob mice, it induced a significant decrease in the α-synuclein ($p<0.05$) (Fig. 7a) and phosphorylated Tau protein expression to the levels detected in wild-type mice ($p<0.001$ for Tau p-S^{199}, $p<0.001$ for Tau p-T^{205}, $p<0.001$ for Tau p-S^{396} and $p<0.001$ for Tau p-S^{404}) (Fig. 7b).

In summary, based on the results presented in this section, leptin deficiency-induced obesity increases the accumulation of neurodegeneration markers, mainly markers of tauopathies, and melatonin is able to restore the normal levels of these markers.

Leptin-deficient mice exhibit distinct stress-induced behaviors

All previous alterations and failures at the cellular level observed in the brains of ob/ob mice could have behavioral implications and may be correlated with behavioral changes in response to an acute stressful and aversive situation. Therefore, we evaluated the possible stress-induced behaviors using the PST. Our results showed significant differences between ob/ob and wild-type mice, as the leptin-deficient mice exhibited lower immobility latency times ($p<0.001$) (Fig. 8a). This increase in mobility revealed an escape behavior that suggested a lower state of hopelessness in ob/ob animals than in wild-type mice (Fig. 8b). The melatonin treatment did not alter the stress-induced behaviors of wild-type animals, but it reversed this behavior in leptin-deficient mice by reducing mobility and increasing immobility to the levels observed in wild-type animals ($p<0.001$) (Fig. 8).

Thus, the cellular alterations detected in the brains of leptin-deficient mice induce changes in behaviors by increasing the animals' perception of stress and probably their susceptibility to anxiety.

Discussion

Obesity, a medical condition that has reached epidemic levels worldwide, is a key factor in several widely studied diseases, such as bone and muscle diseases, heart diseases and type 2 diabetes [57]. Although obesity has been shown to affect the organism from the biochemical level to the tissue level, its effects on the brain, particularly the impact of obesity-induced damage on the brain, have been poorly studied. Therefore, we studied the effects of obesity on cellular mechanisms and showed that obesity induced widespread brain effects that may underlie neurodegenerative disorders ultimately compromising the behavior. Association between obesity and dementia risk are being studied [4].

Morbid obesity, such as is induced in ob/ob mice by leptin absence [58], induced a significant decrease in brain weight in our animals, even though the net body weight of the ob/ob mice was significantly increased compared to the wild-type mice. A similar decrease in brain volume is usually observed during aging and is related to changes in cognition [59]. This concomitant decrease may be the first evidence of cerebral damage.

The decrease in brain volume is not the only similarity between the effects of obesity and aging on the brain. Oxidative stress occurs in aged brain [60,61], and increased food intake causes the excessive oxidation of glucose and exacerbates ROS production [62]. In our obese animals, these changes were manifested as a significant increase in protein damage that was not reduced by GSSG-Px/GR. The tandem dysregulation of SOD and CAT activities magnifies this damage. As shown in aged brains, melatonin was able to counteract this damage and significantly reduces brain damage by restoring and increasing the antioxidant activities of SOD and CAT [63,64].

The oxidative stress observed in the brains of our obese animals dysregulates inflammation by inducing a clear increase in the IL-6 levels and a decrease in the TNF-α levels. IL-6 and leptin are adipokines, and both proteins, together with their receptors, share structural and functional similarities in regulating the immune response [65]. Thus, IL-6 expression is increased in response to oxidative stress, generating a protective anti-inflammatory effect that, in turn, inhibits TNF-α production [66]. The melatonin treatment enhanced this protective effect of IL-6 on both obese and wild-type animals. Likewise, the

slight increase in TNF-α production observed in melatonin-treated obese mice may result from melatonin-induced improvements in the immune system, consistent with several previous reports [67-69]. These data agree with the decrease in the levels of this cytokine in obese animals, where the close relationship between leptin and TNF-α must be assessed [65], since both cytokines exert synergistic effects and in the absence of one cytokine, the levels of the other may be reduced. Nevertheless, TNF-α reduces food intake and participates in regulating energy homeostasis [70]; in the absence of leptin and the presence of reduced TNF-α levels, energy homeostasis is expected to be deregulated, suggesting that the protein synthesis and degradation pathways are impaired.

According to previous studies, including studies from our own group, obesity induces ER stress in different organs [13,66-68]. However, the UPR in the brain has specific and dangerous consequences, since oxidative stress and protein misfolding play critical roles in the pathogenesis of neurodegenerative diseases [71-73] and dementia [74]. The ER has an essential role in neurotransmitter synthesis; therefore, ER stress implies that protein folding efficiency is decreasing, which may contribute to abnormalities in the neuronal circuitry that may represent a preliminary stage of neurological disorders [75]. Oxidative stress and ER stress are observed in the brains of obese animals, as shown by the changes in the main signaling cascades. However, the signals required to trigger these pathways are missing. The primary target of IRE-1α/XBP1 activation is the 26S proteasome [73] to reduce the levels of misfolded proteins, but obese animals showed decreased 20S proteasome activity. Likewise, ATF-6α is cleaved to yield a fragment that migrates to the nucleus to activate the transcription of chaperone genes [76], but the levels of hsc70, one of the most important chaperones that integrates in the hsp70 system and is responsible for preventing aggregation and remodeling folding pathways [48], are significantly reduced in obese animals. Although the UPR is correctly triggered by the misfolded proteins, subsequent transcriptional activation is not produced. These obesity-induced impairments are consistent with data observed in aging models [77], as the ability to activate the UPR decreases with age. The significant increase in the levels of ubiquitinated proteins observed in the obese animals indicates that ER stress is not being resolved. Proteasome dysfunction has been widely documented in obese subjects [78,79]; however, this study is the first to show the deterioration of the UPR signaling cascade in obese animals.

The treatment with melatonin induced a total recovery. Accumulating data support the hypothesis that protein folding and the generation of free radicals in the ER as byproducts of protein oxidation are closely associated events [73]. Under these circumstances, the action of potent antioxidants, such as melatonin, is very beneficial. A minimal UPR was observed in obese animals treated with melatonin because the production of misfolded proteins was drastically reduced; these data were corroborated by the substantial reduction in the levels of ubiquitinated proteins in this experimental group.

Degradation of misfolded proteins only occurs through the proteasome or autophagy. Both CMA and macroautophagy were up-regulated in our obese animals. This situation reduced the levels of misfolded proteins, although misfolded proteins were still present, as shown in the blots of the ubiquitinated proteins. However, this *status quo* cannot be maintained. Thus, Ignacio-Souza and coworkers [79] showed that prolonged obesity, but not short-term, trying to maintain rate of protein recycling, induced eventually ubiquitin/proteasome and autophagy fails. Similar data were observed in our unpublished data from human muscles obtained from overweight aged people [80]. Thus, sustained autophagy fails to remove all damaged structures and directs the cell to programmed cell death. The significant increase in the p62 levels observed in the obese animals showed that autophagy was not as efficient as needed.

The melatonin treatment, which reduces ER stress and oxidative stress in the cell, reduces the need for autophagy, and thus autophagy was significantly reduced in this experimental group. However, the increased p62 levels observed in control animals treated with melatonin may be noteworthy. P62 is a multifunctional molecule and the meaning of this result requires additional information. Therefore, considering the data from the ER stress, oxidative stress, UPR cascade and autophagy experiments, the most likely role of p62 is to counteract oxidative stress and enhance cell protection by interacting with nuclear factor-erythroid 2-related factor 2 (Nrf2), as suggested by some authors [52,81]. Thus, our results about p62 in the treated wild-type animals are probably related with the antioxidant properties of melatonin.

Aggresomes are inclusion bodies composed of misfolded proteins that are aggregated in a single structure mainly in the central region of the cell [54]. However, several other molecules can be also incorporated in aggresomes by synergistic actions. Thus, several cytoskeleton components are present in aggresomes as a result of cytoskeletal disruptions, including intermediate filaments [53], filamentous

actin [82] and, mainly in the central nervous system, microtubules, which have dangerous consequences regarding the transport of neurotransmitters [83]. These aggresomes are also enriched in ubiquitin, a marker of misfolded proteins, and p62, as this molecule acts a bridge between ubiquitin-protein aggregates and the autophagosomal system [83]. Inefficient autophagy leads to the accumulation of autophagosomes enriched in p62 and misfolded/unfolded proteins that assist in the construction of aggresomes. In obese animals, the levels of p62 and misfolded proteins were increased. Cytoskeletal rearrangements are also evident, based on the significant increase in the levels of actin, tubulin and vimentin, supporting the presence of aggresomes. The intermediate filament vimentin is redistributed during aggresome formation to form a cage surrounding a pericentriolar core composed of ubiquitinated proteins [55]; therefore, the increase in the vimentin levels should be related to aggresome formation. Aggresomes gain special importance in obese animals when the brain is the target, since they play a main role in brain deterioration and directly interact with the hyperphosphorylated Tau protein observed in these animals, which also forms part of the aggresome [84].

Tau is a highly soluble protein with multiple phosphorylation sites [85]. Its microtubule binding activity is mainly regulated by phosphorylation, and microtubule assembly is promoted by this posttranslational modification, which stabilizes the microtubule network. However, Tau hyperphosphorylation reduces its capability to bind microtubules [86] and is present in a large number of neurodegenerative disorders known as tauopathies [87]. Likewise, filamentous actin interacts with Tau proteins in inclusion bodies in AD [54,82]. Phosphorylation at four Tau sites was significantly increased in obese animals and are included in the 30 phosphorylation sites observed in the brain of AD patients [87], indicating that obesity may be implicated in tauopathies development. Together with these neurodegenerative markers, the α-synuclein levels were also increased in ob/ob animals. Its accumulation seems to be induced by the oxidative environment, misfolded proteins, accelerated aggregation and impairments in degradation systems, which contribute to the pathogenesis of synucleinopathies [83,88].

The melatonin treatment produced a drastic effect on α-synuclein accumulation, hyperphosphorylation of Tau and aggresome formation. Melatonin efficiently protects neuronal cells and attenuates Alzheimer-like Tau hyperphosphorylation via its antioxidant properties [87]. In our study, melatonin significantly reduced phosphorylation at the four Tau sites studied to the basal level, inhibiting the

development of cognitive deficits associated with some tauopathies [89]. Regarding aggresome formation, the effect of melatonin seems to be mediated by a cascade consisting of reduced oxidative stress and minimized ER stress to resolve protein misfolding. Thus, autophagy overactivity is not required to avoid aggresome formation. Moreover, various authors have revealed the importance of microtubules in the formation and clearance of aggresomes [54,84]; therefore, a significant reduction in α-tubulin expression in the ObM group is expected to decrease the number of these inclusion bodies. This effect of melatonin as an inducer of a mechanism preventing aggresome formation was previously unknown.

Tauopathies [90] and synucleinopathies [91] have been described to induce cognitive deficits. Consequently, all cellular damage observed in the brains of obese animals, together with Tau hyperphosphorylation and α-synuclein accumulation, could promote abnormal behaviors. To test this hypothesis in our experimental model, we used the PST that measure anxiety- and/or depression-related behaviors since these behaviors are common and prodromal symptoms of neurodegenerative diseases [92-95]. Although the PST allows us to study depression-related factors, the PST is not a sufficient screen for depression.

Although several studies have correlated low leptin levels in the plasma or cerebrospinal fluid with a risk of major depression (REFs), leptin deficiency-induced obesity increases mobility latency times, suggesting an increase in the animals' perception of stress and anxiety [96,97]. On the other hand, the accumulation of abnormally hyperphosphorylated Tau proteins increases anxiety-related behaviors [98,99].

As discussed above, melatonin significantly reduces the negative effects of obesity on the brain, which may be sufficient to explain the recovery in stress-induced behaviors until control levels observed in melatonin-treated obese mice, which presented similar mobility latency times than wild-type animals. Moreover, anxiolytic and antidepressant-like effects were attributed to melatonin [95,100]. These multifactorial actions of melatonin are certainly the reason for the evident improvements in the behaviors of the treated obese animals.

In summary, morbid obesity, which was induced by a leptin deficiency in the ob/ob animals, provokes significant alterations in the brain at cellular level that altered their behaviors. Oxidative stress is

accompanied by ER stress in the brains of these animals. Thus, UPR and protein degradation systems (proteasome, CMA, autophagy, etc.) begin to be overwhelmed, aggresomes are formed, and neurodegenerative markers are expressed, all of which are also present in non-pathological aging. Aggresomes always have harmful effects on cell transport, but they are particularly deleterious to postmitotic neurons, which require neurotransmitter transport and have no capacity to dilute protein aggregates by cell division, leading to cell death. Finally, these alterations increase the animals' perception of stress and anxiety.

Melatonin improves the antioxidant defense system by reducing the levels of damaged and unfolded/misfolded proteins, thus attenuating the failure of the protein repair and degradation systems and decreasing the levels of protein aggregates and brain damage caused by obesity. Additionally, the melatonin treatment decreases the stress-induced behaviors and anxiety. Therefore, our results suggest that melatonin is a potential therapeutic agent that protects against brain damage in obesity specifically tested.

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Conflict of interest

The authors declare that they have no conflict of interest.

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882
883

884 # Table footnotes

885 **Table 1** The data are shown as the mean ± SD, and were calculated from at least three separate
886 experiments performed in triplicate. WC, wild-type; WM, wild-type plus melatonin; ObC, ob/ob; ObM,
887 ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated animals vs. melatonin-untreated
888 animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.

889

890 # Figure captions

891 **Fig. 1** Oxidative damage and antioxidant defense in the brain of wild-type and ob/ob mice. (a) Protein
892 damage was expressed as nmol carbonylated protein/mg protein. (b) Total antioxidant activity (TAA)
893 was expressed as equivalents of mg equivalents Trolox/mg protein. (c) Superoxide dismutase (SOD)
894 activity was expressed as SOD units/mg protein. (d) Catalase (CAT) activity was expressed as μmol
895 H_2O_2/mg protein*min. (e) Glutathione peroxidase (GPx) activity was expressed as μmol NADPH/mg
896 protein*min. (f) Glutathione reductase (GR) activity was expressed as μmol NADPH/mg protein*min.
897 The data are presented as the mean values ± standard deviations (SD) of the means. All data presented
898 representative data obtained from at least three separate experiments. WC, wild-type; WM, wild-type
899 plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated
900 animals vs. untreated animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.

901

902 **Fig. 2** Inflammatory markers from the brain of wild-type and ob/ob mice. Tumour necrosis factor α
903 (TNF-α) and interleukin 6 (IL-6) levels were expressed as pg/mg protein. The data are presented as the
904 mean values ± standard deviations (SD) of the means. All data presented representative data obtained
905 from at least three separate experiments. WC, wild-type; WM, wild-type plus melatonin; ObC, ob/ob;
906 ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated animals vs. untreated
907 animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.

908

909 **Fig. 3** Endoplasmic reticulum (ER) stress markers in the brain of wild-type and ob/ob mice. (a) Bar
910 chart showing the semiquantitative optical density (arbitrary units of blots bands) of inositol-requiring

enzyme 1α (IRE1α), phosphorylated-eukaryotic initiation factor 2α (p-eIF2α) and activating transcription factor-6α (ATF-6α) from western blot normalized to ponceau and expressed as percent change from the WC sample. (b) Bar chart showing the semiquantitative optical density (arbitrary units of blots bands) of heat shock cognate 71 kDa (hsc70) protein from sodium dodecyl sulfate-polyacrylamide gel image of protein extracts of the brain obtained from analysis by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) mass spectrometry. The results were expressed as percent change from the WC sample. The data are presented as the mean values ± standard deviations (SD) of the means. All data presented representative data obtained from at least three separate experiments. WC, wild-type; WM, wild-type plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated animals vs. untreated animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.

Fig. 4 Ubiquitin-Proteasome system in the brain of wild-type and ob/ob mice. (a) 20S proteasome activity is expressed as arbitrary fluorescence μM AMC/mg protein. (b) Bar chart showing the semiquantitative optical density (arbitrary units of blots bands) of ubiquitin from western blot normalized to ponceau and expressed as percent change from the WC sample. The data are presented as the mean values ± standard deviations (SD) of the means. All data presented representative data obtained from at least three separate experiments. WC, wild-type; WM, wild-type plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated animals vs. untreated animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.

Fig. 5 Autophagy markers in the brain of wild-type and ob/ob mice. Bar chart showing the semiquantitative optical density (arbitrary units of blots bands) of lysosome associated membrane protein type 2A (LAMP2A), Beclin-1, microtubule-associated protein 1 light chain 3 I (LC3-I), microtubule-associated protein 1 light chain 3 II (LC3-II) and p62 from western blot normalized to ponceau and expressed as percent change from the WC sample. The data are presented as the mean values ± standard deviations (SD) of the means. All data presented representative data obtained from at least three separate experiments. WC, wild-type; WM, wild-type plus melatonin; ObC, ob/ob; ObM,

939 ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated animals vs. untreated animals.
940 #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.
941
942 **Fig. 6** Cytoskeleton markers in the brain of wild-type and ob/ob mice. Bar chart showing the
943 semiquantitative optical density (arbitrary units of blots bands) of β-actin, α-tubulin and vimentin from
944 western blot normalized to ponceau and expressed as percent change from the WC sample. The data
945 are presented as the mean values ± standard deviations (SD) of the means. All data presented
946 representative data obtained from at least three separate experiments. WC, wild-type; WM, wild-type
947 plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and * melatonin-treated
948 animals vs. untreated animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001, ***p<0.001.
949
950 **Fig. 7** Neurodegeneration markers in the brain of wild-type and ob/ob mice. (a) Bar chart showing the
951 semiquantitative optical density (arbitrary units of blots bands) of β-amyloid and α-synuclein from
952 western blot normalized to ponceau and expressed as percent change from the WC sample. (b) Bar chart
953 showing the semiquantitative optical density (arbitrary units of blots bands) of Tau pS^{199}, Tau pT^{205}, Tau
954 pS^{396} and Tau pS^{404} from western blot normalized to ponceau and expressed as percent change from the
955 WC sample. The data are presented as the mean values ± standard deviations (SD) of the means. All
956 data presented representative data obtained from at least three separate experiments. WC, wild-type;
957 WM, wild-type plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and *
958 melatonin-treated animals vs. untreated animals. #p<0.05, *p<0.05, ##p<0.01, **p<0.01, ###p<0.001,
959 ***p<0.001.
960
961 **Fig. 8** Evaluation of stress-induced behavior in the brain of wild-type and ob/ob mice. Bar chart showing
962 the (a) immobility and the (b) mobility latency time percents in Porsolt Swim Test (PST) for last 4 min
963 of the test. The data are presented as the mean values ± standard deviations (SD) of the means. All data
964 presented representative data obtained from at least three separate experiments. WC, wild-type; WM,
965 wild-type plus melatonin; ObC, ob/ob; ObM, ob/ob plus melatonin. # wild-type vs. ob/ob and *

melatonin-treated animals vs. untreated animals. #$p<0.05$, *$p<0.05$, ##$p<0.01$, **$p<0.01$, ###$p<0.001$, ***$p<0.001$.

Table 1. Body and tissue parameters: body weight and brain weight at sacrifice.

	WC	WM	ObC	ObM
Body weight at sacrifice (g)	24,19 ± 1,55	24,86 ± 2,08	49,93 ± 5,01 ###	51,48 ± 2,10
Brain weight at sacrifice (g)	0,35 ± 0,02	0,35 ± 0,00	0,31 ± 0,03 ##	0,34 ± 0,03

Figure 1

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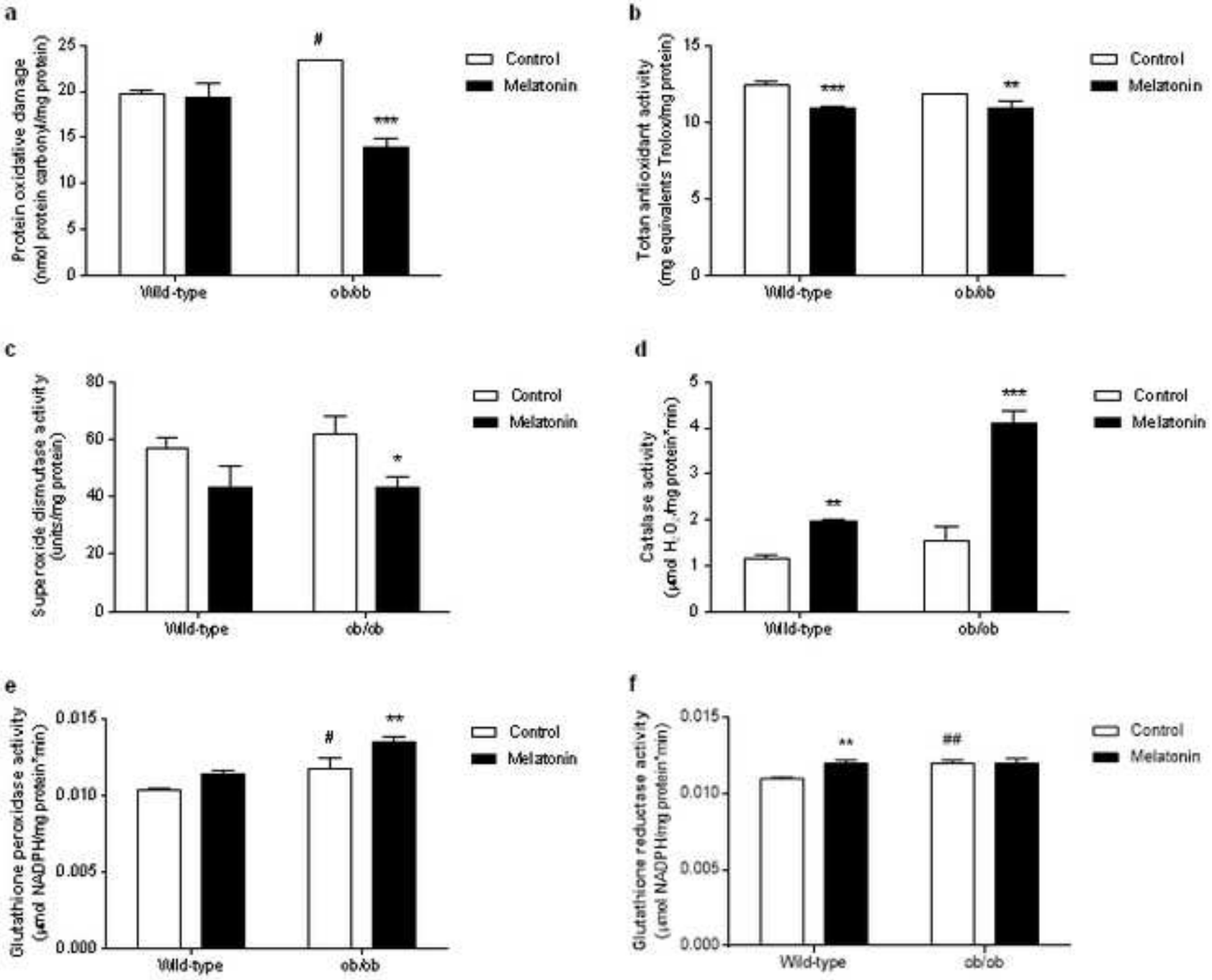

Figure 2

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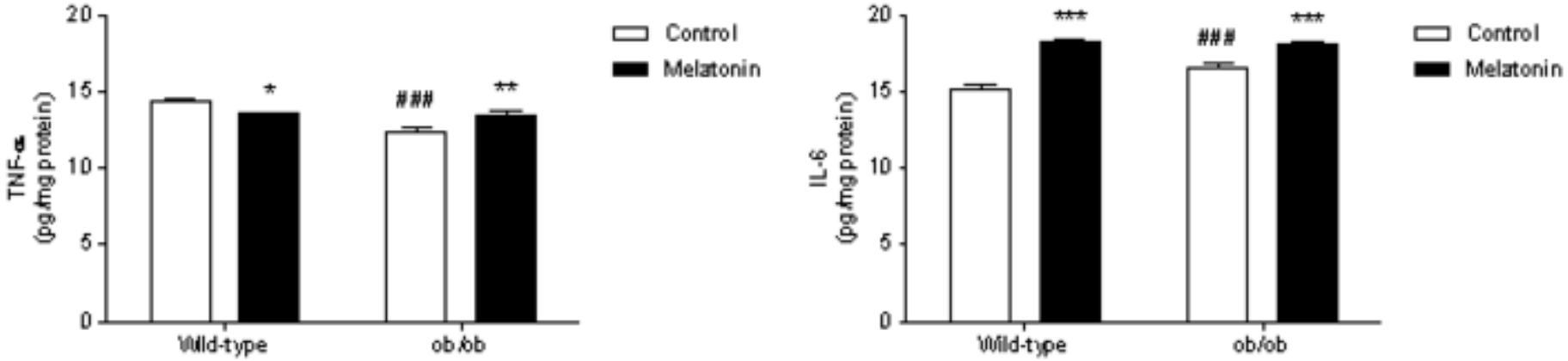

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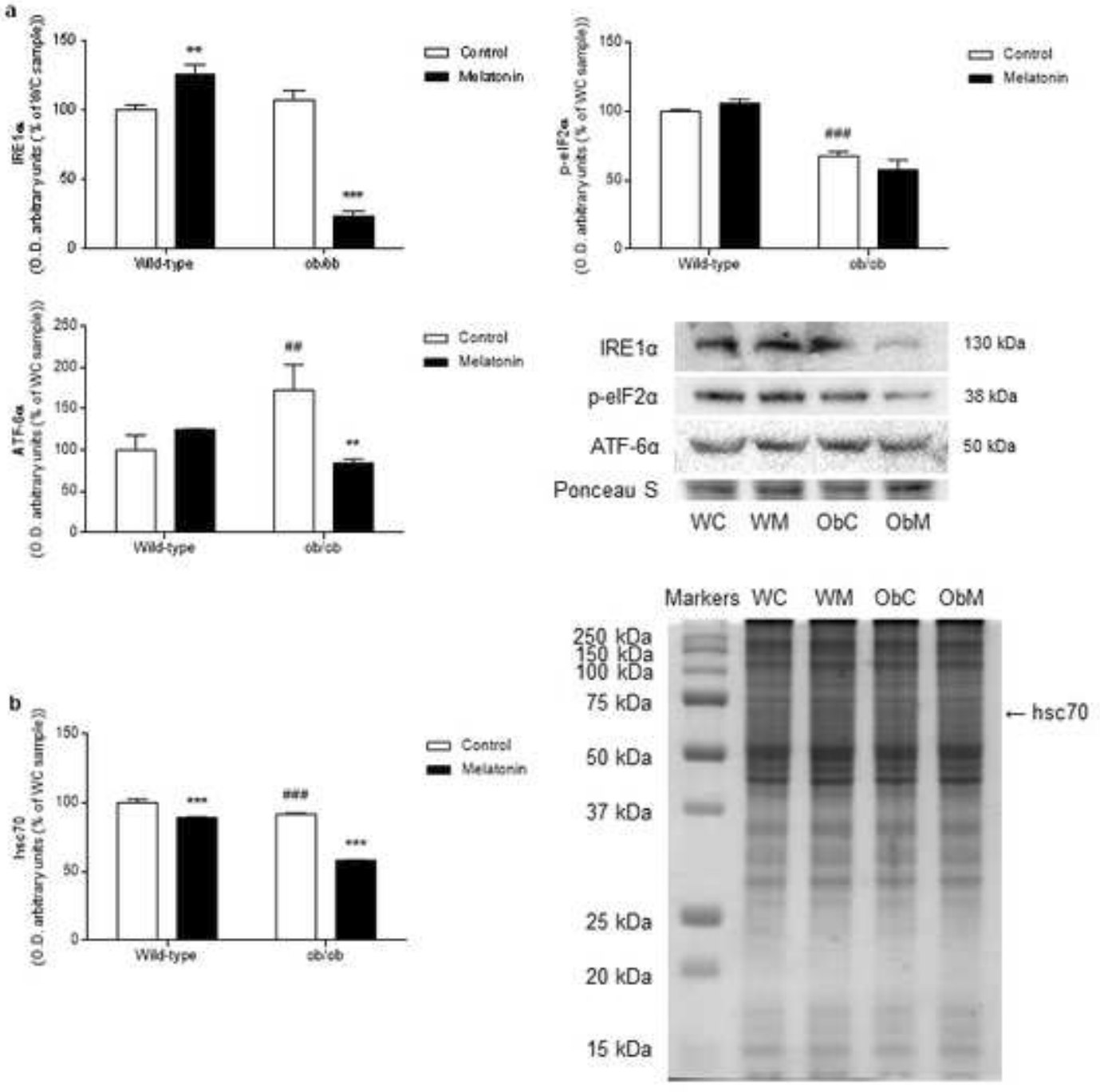

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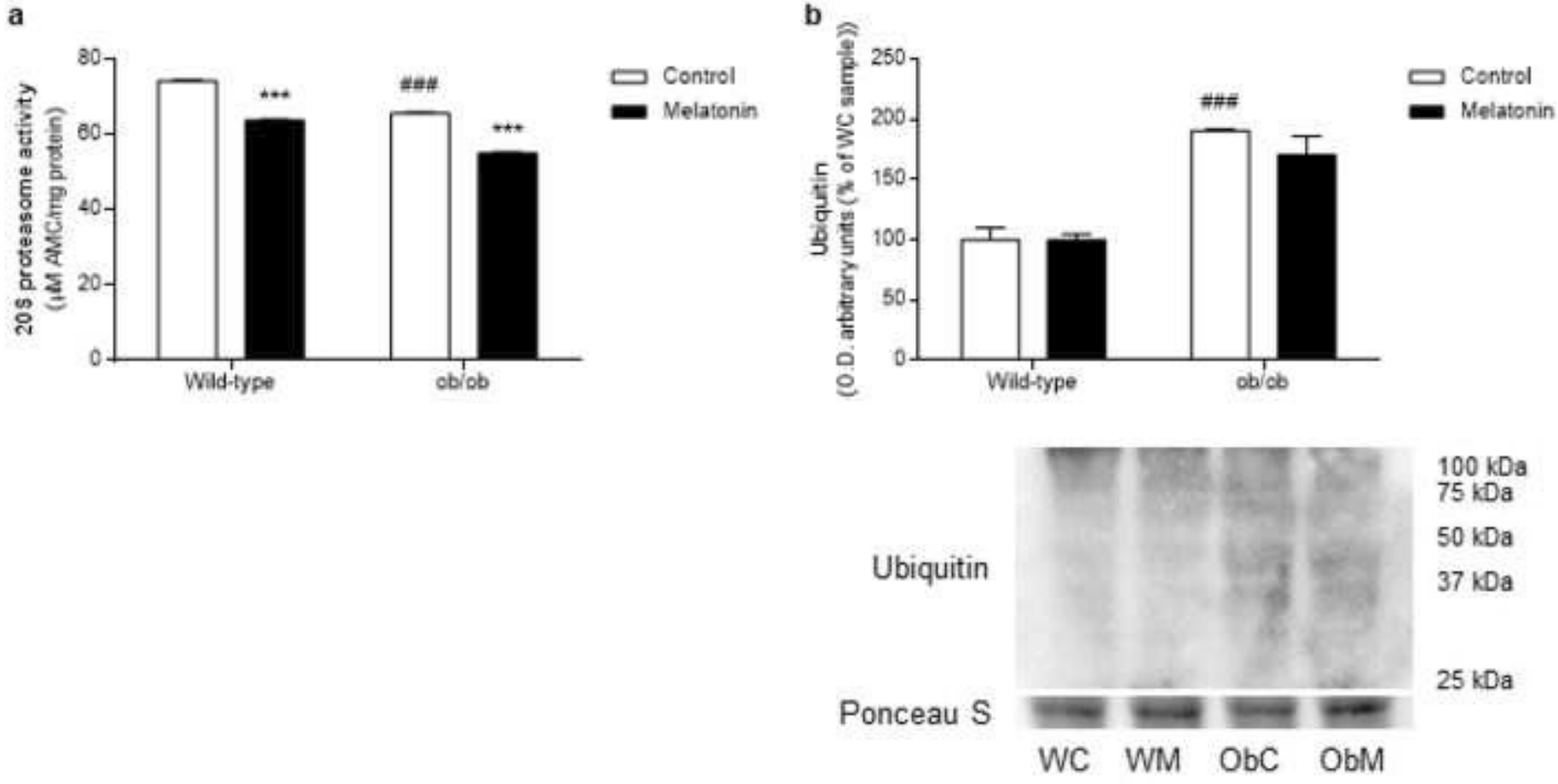

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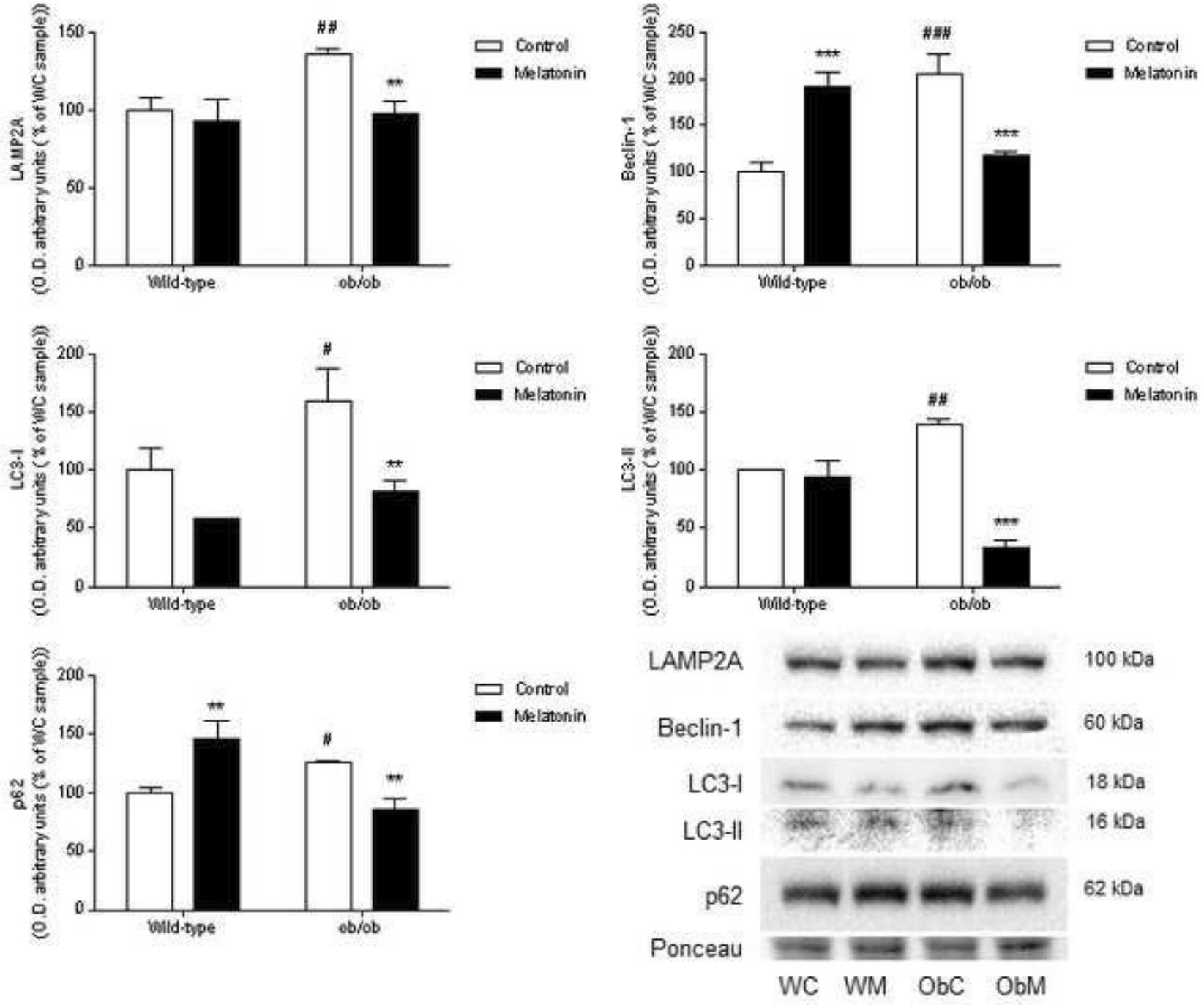

Figure 6

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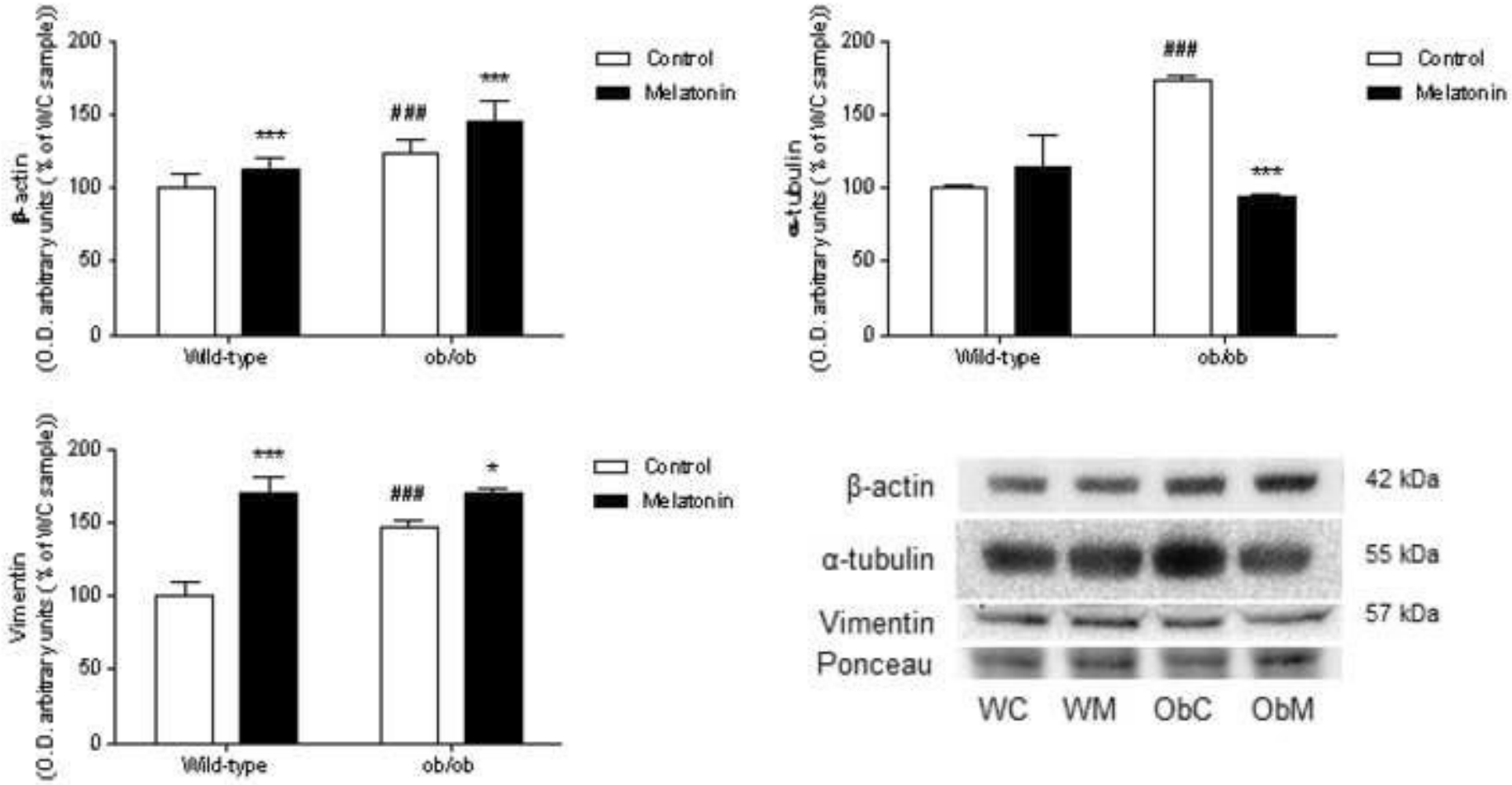

Figure 7

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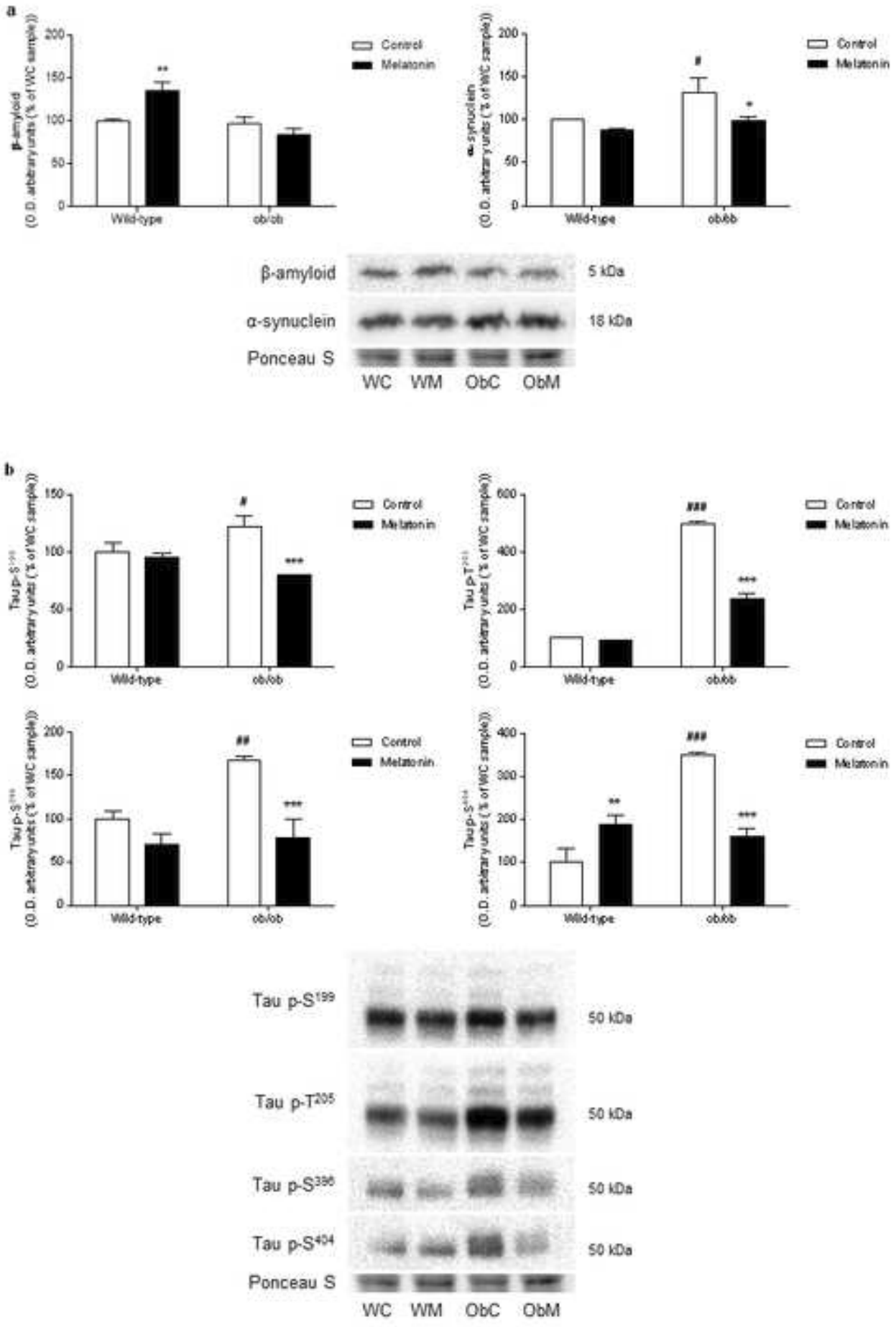

Figure 8

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