Research Article

Pleiotropic role of melatonin in brain mitochondria of obese mice

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ABSTRACT

Obesity induced by a leptin deficiency causes extensive damage in brain due to a large increase in oxidative stress. Mitochondria have a central role in this neural damage. Leptin receptors have a wide expression in the brain and its absence is associated with reduced mitochondrial respiration by a decoupled electron transport chain and a significantly increased complex II activity which is major player in mitochondrial free radical generation. The consequences are an abrupt reduction in ATP production and a reduced activity of the tricarboxylic acid (TCA) cycle, evidence of dysfunction of processes related to energy production, the reduction of which contributes to multiple brain pathologies. Melatonin, a major protector of mitochondria against free radicals with a significant influence on glucose metabolism, has been shown to counteract these conditions. In the present study the main respirasome expression was recovered by melatonin, with a reduction in complex II activity and the complex II dependent free radical generation. Additionally, melatonin normalized the TCA cycle. Reduction in ATP synthesis was caused by UCP2 activation. The uninterrupted sensation of starvation due to leptin deficiency involves impairments in glucose metabolism, which was reversed by melatonin via negatively acting on hexokinase II, a key regulator of glycolysis and major contributor to the Warburg effect. Hexokinase II reduction was accompanied by a significant Bcl-2 reduction, inducing a delicate readjustment of pro and anti-apoptotic proteins in the mitochondria to preserve cell survival, which was associated with a marked reduction of the Bax activator, Puma, observed in obese animals treated by melatonin.

Considering currently lack of treatment on this genetic disease, melatonin may provide an excellent palliative remedy for this situation. .

Key words: Melatonin, leptin deficiency, obesity, brain, brain damage, mitochondria, hexokinase II, autophagy.

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1. INTRODUCTION

Obesity is a multifactorial syndrome and several factors are implicated in its development. This hinders a complete understanding of the mechanisms and, consequently, means to combat it (1). Among these factors, one of the most outstanding is leptin-deficiency due to its pleiotropic effects (2). Leptin is an adipokine not only crucial in weight gain but also a molecule responsible for the regulation of appetite (2) and thermogenesis (3) and endocrine regulation (4). This complexity may be a reason of the limited knowledge about pathological effects caused by a leptin deficiency. When these changes are focused on brain function, the information is further expanded.

Leptin, as with other adipokines, has receptors widely expressed in the brain (5). Evidence indicates that leptin crosses the blood-brain barrier (6) with its signaling being involved in important neuronal processes such as hippocampal neuroplasticity (7) and spatial learning, memory formation and emotional regulation (8). Melatonin, a powerful natural antioxidant, is also involved in neuroendocrine processes (9, 10) and has been shown to reduce body mass under diverse obesity situations (11, 12). Its interactions with leptin are multiple, i.e., both hormones are essential in the regulation of energy balance and body mass, exhibit analogous circadian rhythms and adipocytes, where leptin is synthetized, have melatonin receptors (13, 14). Moreover, a melatonin deficiency induces leptin resistance in rats, while melatonin treatment restores leptin sensitivity (15); this implies that melatonin directly interacts with leptin. In the brain, where leptin acts as afferent signaling molecule and melatonin functions as the neuroprotector (16, 17) even during obesity (18). Previous data from our laboratory have shown the beneficial role of melatonin in leptin deficiency-dependent obesity (19-21).

Obesity, itself, is an major contributor to brain damage by increasing the number of fat cells that damage white matter and trigger vascular dementia (22). A high-fat diet likewise induces important alterations in the cytoskeleton and synaptic plasticity (23). Moreover, a leptin deficiency enhances the already deleterious effects that obesity provokes in the brain at molecular and cellular levels (19) since it induces an uninterrupted sensation of starvation. This involves impairments in glucose metabolism and in mitochondrial function as primary changes that gain additional importance in the brain (22, 23). Both processes, glucose metabolism and mitochondrial function, have close link to hexokinase II (HKII). HKII is the enzyme catalyzes glucose into glucose-6-phosphate in glycolysis. It is the first rate-limiting enzyme in this pathway and this makes HKII a key regulator of glycolysis (24, 25). Moreover, HKII also relates to mitochondria function (25). Via mitochondrial binding motif, HKII attaches to the outer mitochondrial membrane (mitoHKII) and this allows it to establish a connection between glycolysis and oxidative phosphorylation (26). Excessive nutrient supply, common in obesity, causes important mitochondrial dysfunction by overwhelming the citric acid cycle and the electron transport chain (27). Mitochondria, in turn, can manage this excessive ATP synthesis induced by high glucose consumption utilizing uncoupling proteins (UCP). UCPs are in charge of energy dissipation by heat generation in mitochondria (28, 29). Located in the mitochondrial inner membrane, UCPs dissipate the proton gradient generated in mitochondrial intermembrane space by regulating proton channels, thereby, moderately

reducing ATP production. UCP2 activity seems to influence glucose homeostasis by fine tuning intracellular events such as controlling insulin secretion or food intake behavior (30).

HKII has a role on mitochondria protection (25, 31). Leptin, as an afferent signal to the brain, regulates energy homeostasis and modulates mitochondrial physiology in non-neural tissues (32) but its absence may aggravate cerebral damage. And, classically, melatonin is considered essential molecule in the preservation and modulation of mitochondria (33-35).

These three molecules, i.e., HKII, leptin and melatonin, collaborate in multiple cellular processes. Thus, HKII also to plays a crucial role in cell survival (24, 25, 31) as a protective molecule against oxidative stress (26) and the induction of autophagy (24), including mitophagy, a selective mitochondrial autophagy, that occurs when oxidative stress increases or when mitochondrial dysfunction is exacerbated. Likewise, HKII is also involved in the regulation of apoptotic processes; several studies have demonstrated that HKII competes with apoptotic B-cell lymphoma 2 protein (Bcl-2) family proteins, such as Bax, to prevent mitochondrial outer membrane rupture (36, 37). Conversely, leptin may have beneficial effects against oxidative stress (38) and in modulating autophagy (39). Alterations in apokines including leptin and adiponectin suppress autophagy and promotes cellular senescence (40). Leptin also promotes cancer cell apoptosis (41). The roles of melatonin as an autophagy regulator (42, 43) and modulator of apoptosis (44) are well documented.

Mitochondral alterations occur in the brain during obesity but their damage is not well defined. The coincidence, well established, between obesity and chronic oxidative stress (45), including in the brain (46), supports changes in mitochondrial function. Although melatonin is considered an essential molecule in the preservation of mitochondria, its role in brain energy balance in obesity in general and in morbid obesity induced by leptin resistance, in particular, is also not well known.

In the current research, focus is on mitochondrial function under the multifactorial situations presented in ob/ob mice. We have observed that in the genetic obesity mice, their brain mitochondria are seriously degraded and melatonin is the only molecule capable of significantly reducing the damage. The potential mechanisms are that melatonin modulates mitochondrial and glucose metabolisms caused by the absence of leptin in the brain.

2. MATERIALS AND METHODS

2.1. Animals.

Twenty-eight six-week-old male wild-type (C57BL/6J) and twenty-eight age matched male leptin-deficient obese (B6.V-Lepob/J) (ob/ob) mice were purchased from Charles River (Charles River Laboratories España, SA, Barcelona, Spain). The mice were housed two per cage under 12:12 hours dark:light cycle at 22 ± 2°C. The animals received tap water and a standard chow diet *ad libitum*.

2.2. Melatonin treatment.

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

At 2 hours after lights off, intraperitoneal injections of melatonin (Sigma-Aldrich, St Louis, MO, USA) were administered daily at a dose of 500 µg/kg body weight for 4 weeks. Melatonin was dissolved in 0.5% absolute ethanol:saline. Animals in the control groups received vehicle at comparable amount, route and treatment duration.

The experiment 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). The protocol

2.3. Tissue parameters.

The brains from all the animals were weighed at the end of the experiment (sacrifice).

2.3.1. Mitochondria isolation.

Twenty-four animals (six mice per group) were sacrificed by decapitation, and the brain of each mouse was immediately removed. Mitochondria isolation was performed following the protocol of Oliveira and Silva (47). Then, mitochondria were stored on ice until initiating the oxygen respiration assays, and their protein content was determined by Bradford method (48).

2.3.2. Oxygen consumption.

Oxygen consumption was measured in a suspension of freshly isolated mitochondria at 37 °C and monitored polarographically with a Clark-type oxygen electrode (Oxygraph, Hansatech Instruments Ltd, Pentney, UK), following the protocol of Oliveira and Silva (47).

The desired volumes of respiration buffer (135 mM sucrose, 65 mM KCl, 5 mM KH_2PO_4, 5 mM HEPES, and 2.5 mM $MgCl_2$ (pH 7.4)) and mitochondrial suspension (1mg/mL) were introduced into the chamber. Energization was achieved with 10 mM glutamate + 5 mM malate (G/M) followed by 175 nmol ADP, or with 5mM succinate (SUC) in presence of 1 µM rotenone followed by 125 nmol ADP. When all ADP was consumed, 1 µg oligomycin was added to determine oxygen consumption with inhibited ATP synthase.

The oxidation rates were expressed in nanoatoms of oxygen consumed per minute per milligram of protein (natom oxygen/min*mg protein). Respiratory control ratio (RCR), i.e., the ratio of the state 3 to state 4; and the oxidative phosphorylation (OXPHOS) efficiency (ADP/O ratio), i.e., as the ratio between the amount of ADP added (in nmol) and the oxygen consumed (in natom) during state 3, were also calculated.

2.3.3. Tissue processing.

Thirty-two animals (eight mice per group) were sacrificed by decapitation, and the brain of each mouse was immediately removed, flash-frozen with liquid nitrogen and stored at -80°C until further analysis.

The brain of each mouse was homogenized at a ratio 1:10 (w:v) in homogenization buffer (pH 7.5) containing 50 mM sodium phosphate buffer, 100 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF and 1% Triton using an Ultra-Turrax T25 Mixer (IKA, Staufen, Germany). The homogenates were centrifuged at 900 g for 6 min at 4ºC. Supernatants containing proteins were collected, aliquoted and frozen at -80ºC until further analysis. The Bradford method was used to quantify protein amount in the brain supernatants (48).

2.3.4. Western blot immunoassay.

The tissue homogenates (25 or 50 µg of protein per sample) were mixed with Laemmli sample buffer (BioRad Laboratories, Inc., Hercules, CA, USA) and denatured by boiling at 100°C for 5 min. The samples were fractionated using SDS-polyacrylamide gel electrophoresis

(PAGE) at 200 V and subsequently transferred onto a polyvinylidene fluoride sheets (PVDF) at 350 mA (Immobilon TM-P; Millipore Corp., Bedford, MA, USA).

The membranes were blocked for 1 hour at room temperature with 10% (w/v) nonfat dry milk dissolved in Tris-buffered saline (TBS) (50 mM Tris/HCl, pH 7.5, 150 mM NaCl). Subsequently, the membranes were incubated overnight at 4ºC with the respective primary antibodies: MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam, Cambridge, UK), anti-Tom20 (42406, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-UCP2 (89326, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-Hexokinase II (C64G5, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-Nix (N0399, Sigma-Aldrich, St Louis, MO, USA), anti-BNIP3 (3769, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-Mitofusin-2 (9482, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-DRP1 (8570, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-Bcl-2 (3498, Cell Signaling, Merck Millipore, Billerica, MA, USA, anti-Bax (5023, Cell Signaling, Merck Millipore, Billerica, MA, USA), anti-Bad (9239, Cell Signaling, Merck Millipore, Billerica, MA, USA), and anti-Puma (12450, Cell Signaling, Merck Millipore, Billerica, MA, USA) previously diluted in TBS buffer containing 1% (w/v) nonfat dry milk and 0.04% sodium azide. After three 10 min 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 (Sigma Aldrich, Saint Louis, MO, USA) diluted in TBS buffer containing 1% (w/v) nonfat dry milk for 1 hour at room temperature, followed by three 10 min washes in TBS-T.

The membranes were developed using a chemiluminescent substrate (WBKLS0500, Merk Millipore, Billerica, USA) according to the manufacturer's protocol. The levels of proteins were quantitatively analyzed using Image Studio Lite 5.0.21 software (LI-COR Biotechnology, Lincoln, NE, USA). The results were normalized to Ponceau S as loading control and relative to the WC sample.

2.3.5. Protein identification by peptide mass fingerprint.

Brain protein homogenates (15 μg) were mixed with Laemmli sample buffer (BioRad Laboratories, Inc., Hercules, CA, USA) and boiled at 100°C for 5 min to complete protein denaturation. Both samples and prestained molecular weight standards (Precision Plus Protein All Blue Standards (BioRad Laboratories, Inc., Hercules, CA, USA)) were loaded to 10% SDS-polyacrylamide gel. Gels were run at 100 V and stained in a mixture of 30% (v/v) methanol, 10% (v/v) acetic acid and 0.01% (w/v) Coomassie Brilliant Blue R-250 (BioRad) and destained using a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid. Stained gel images were captured using a GS-800 Imaging Densitometer (Bio-Rad) and semiquantitatively analyzed using Image Studio Lite 5.0.21 software (LI-COR Biotechnology, Lincoln, NE, USA). The results were relative to the WC sample.

The processing of the bands of interest was conducted following the protocol described by Oliván and collaborators (49) and our research group (19) for its analysis in matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).

2.3.6. Bioluminescent.

Adenosine Triphosphate (ATP) brain levels were determined using a commercially available ATP bioluminescent kit (FLAA, Sigma-Aldrich, Saint Louis, MO, USA). The assay was performed as indicated by the manufacturer. The results are expressed as nmol ATP/g protein.

2.4. Statistical analysis.

All results are expressed as mean ± standard deviations (SD). All data presented are representatives from at least three separate experiments. The results were analyzed via two-way ANOVA, with a model being the first effect the phenotype (WC and ObC) and the second one the treatment (WC and ObC, 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).

3. RESULTS

3.1. Leptin deficiency-induced obesity reduces brain weight.

Leptin-deficient mice showed lower brain weight compared with wild-type animals ($p<0.001$) while melatonin treatment increased brain weight to similar values of wild-type animals ($p<0.05$) (Figure 1).

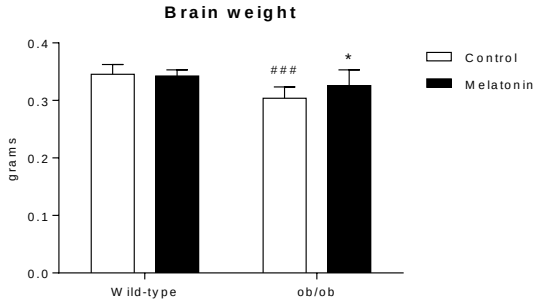

Fig. 1. Effects of melatonin and leptin deficiency on the brain weight (in grams) of wild-type and ob/ob mice.

*All data presented are 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.*

3.2. Leptin-deficient mice present lower synthesis of mitochondrial electron transport chain complexes.

We hypothesized that OXPHOS machinery was remodeled in leptin-deficient mice; thus, the expression of subunits of the mitochondrial electron transport chain complexes were evaluated, namely NADH dehydrogenase (ubiquinone) 1 β subcomplex 8 (NDUFB8) from complex I; succinate dehydrogenase (ubiquinone) iron-sulfur subunit (SDHB) from complex II; ubiquinol-cytochrome c reductase core protein II (UQCRC2) from complex III; cytochrome c oxidase subunit I (MTCO1) from complex IV and ATP synthase subunit α (ATP5A) from complex V by immunodetection. Leptin-deficient animals presented a lower synthesis of protein content of subunits from complexes I, III, IV and V compared with wild-type mice ($p<0.001$ for complex I; $p<0.001$ for complex III; $p<0.001$ for complex IV; $p<0.001$ for complex V), however, these mice have higher amount of SDHB ($p<0.001$) (Figure 2A-E).

Melatonin treatment decreased the amount of UQCRC2-complex III ($p<0.05$) but did not cause changes in the amounts of subunits from complexes I, II, IV and V in wild-type mice (Figure 2A-E). In contrast, in ob/ob animals, melatonin reduced the amount of subunits from

complexes II and V (p<0.001 for complex II; p<0.001 for complex V) and increased the amount of subunits from complexes I, III and IV (p<0.001 for complex I; p<0.05 for complex III; p<0.001 for complex IV) (Figure 2A-E).

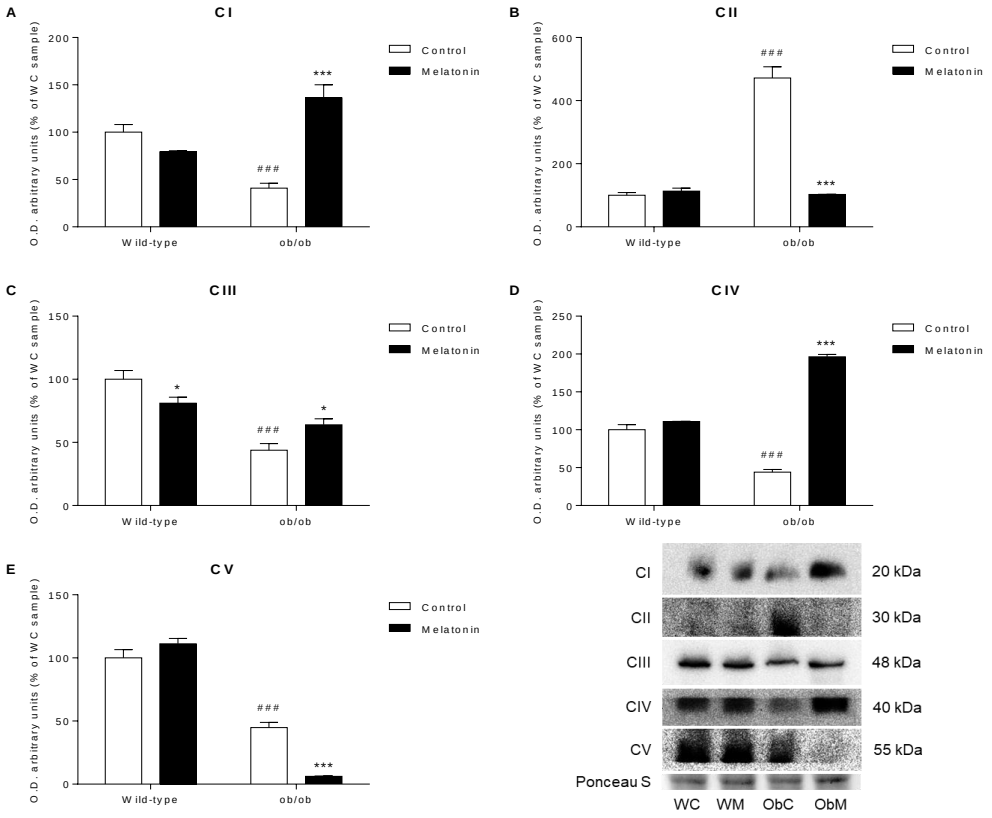

Fig. 2. Effects of melatonin and leptin deficiency on mitochondrial electron transport chain elements in the brain of wild-type and ob/ob mice.

*Bar charts showed the semiquantitative optical density (arbitrary units of blots bands) of (A) NADH dehydrogenase (ubiquinone) 1b subcomplex 8 (NDUFB8) of complex I (CI), (B) iron-sulfur subunit (SDHB) of complex II (CII), (C) the subunit of protein II of the nucleus of ubiquinol-cytochrome c reductase (UQCRC2) of complex III (CIII), (D) subunit I of cytochrome c oxidase (MTCO1) of complex IV (CIV) and (E) the α subunit of the ATP synthase α (ATP5A) of the V-complex from western blot normalized to Ponceau S 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.*

3.3. Leptin deficiency associated obesity decreases mitochondrial function and lowers ATP production.

To evaluate whether this electron transport chain remodeling causes changes in mitochondrial function, the oxygen consumption in isolated mitochondria from brains of both strains of mice was measured. Data obtained from ADP-stimulated respiration (state 3) and respiration in the absence of ATP synthesis (state 4) using glutamate/malate (G/M) as respiration substrates revealed a lower mitochondrial respiration in ob/ob mice than in wild-type mice (p<0.001) (Figure 3A-B). The treatment with melatonin decreased both state 3

(p<0.001 for WC compared with WM and p<0.05 for ObC compared with ObM) and state 4 (p<0.001 for WC compared with WM and p<0.05 for ObC compared with ObM) respiration in both strains of mice (Figure 3A-B). When the respiratory control ratio (RCR) was calculated, a significant decrease in the coupling between substrate oxidation and OXPHOS in leptin-deficient animals (p<0.05) was observed (Figure 3C). The treatment with melatonin increased RCR values in wild-type animals (p<0.001) but did not cause changes in ob/ob mice (Figure 3C). The analysis of the effectiveness of OXPHOS (ADP/O) in the presence of G/M showed a less effectiveness in ob/ob than that in wild-type mice (p<0.001) (Figure 3D). In this case, melatonin produced a significant increase in the efficiency of OXPHOS in ob/ob animals (p<0.01) (Figure 3D).

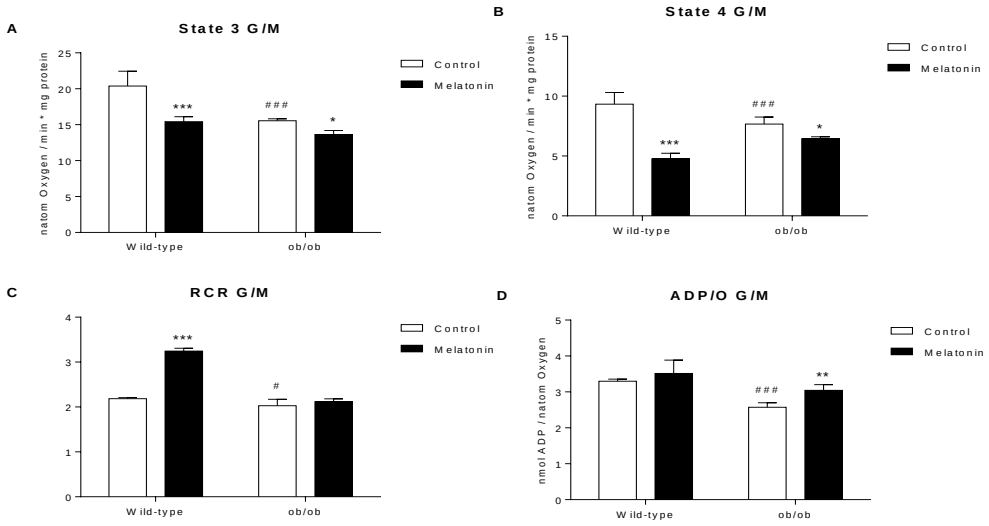

Fig. 3. Effects of melatonin and leptin deficiency on oxygen consumption rate.

*Mitochondrial respiration starts from complex I of the electron transport chain using Glutamate/Malate (G/M) as respiration substrate in the brain of wild-type and ob/ob mice. Bar chart shows (A) the maximum oxygen consumption in the presence of ADP (state 3) and (B) in the absence of ADP (state 4), (C) the respiratory control ratio (RCR) and (D) the efficiency of oxidative phosphorylation (ADP/O). The data are expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD). All data presented are 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.*

When succinate (SUC) was used as substrate for the electron transport chain together with rotenone and therefore without taking into consideration respiration from complex I, ADP-stimulated respiration(state 3) was significantly lower in leptin-deficient mice than in wild-type animals (p<0.001) and no changes were observed in respiration in the absence of ATP synthesis (state 4) (Figure 4A-B). Conversely, melatonin treatment reduced these respiration rates in wild-type mice (p<0.001) while, in ob/ob mice, melatonin did not cause any change in state 3 respiration but lowered respiration in the absence of ATP synthesis (p<0.001) (Figure 4A-B). Leptin-deficient mice showed a lower RCR than wild-type animals (p<0.001) indicating that their mitochondria are uncoupled and melatonin treatment increased this ratio in both genotypes (p<0.001) (Figure 4C). Regarding ADP/O ratio in the presence of SUC, there were no significant differences between ob/ob mice and wild-type animals and melatonin treatment only increased OXPHOS effectiveness in wild-type mice (p<0.01) (Figure 4D).

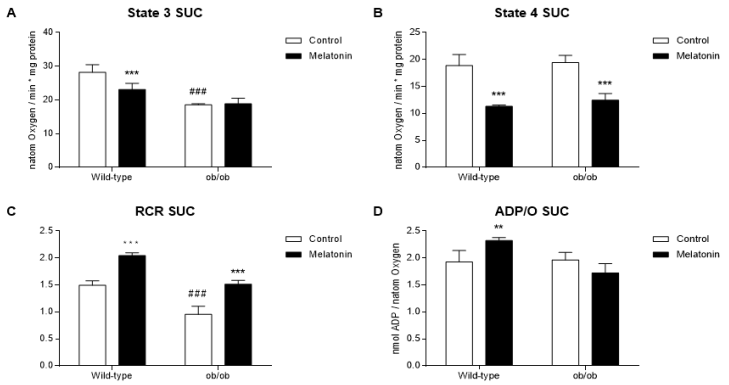

Fig. 4. Effects of melatonin and leptin deficiency on oxygen consumption rate when mitochondrial respiration starts from complex II of the electron transport chain.

*Succinate (SUC) was used as the respiration substrate in the brain of wild-type and ob/ob mice. Bar charts show (A) the maximum oxygen consumption in the presence of ADP (state 3) and (B) in the absence of ADP (state 4), (C) the respiratory control ratio (RCR) and (D) the efficiency of oxidative phosphorylation (ADP/O). The data are expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

After obtaining the results on energy metabolism, we tested whether obesity affects ATP production. For this, mitochondrial and cytosolic ATP levels were measured. It was found that mitochondrial and cytosolic ATP contents were lower in leptin-deficient mice than those in wild-type animals (p<0.001) (Figure 5A-B). The treatment of wild-type mice with melatonin decreased mitochondrial ATP levels (p<0.001) while it maintained cytosolic ATP levels unchanged. In contrast, melatonin treatment in ob/ob mice decreased both mitochondrial and cytoplasmic ATP content (p<0.001 for mitochondrial ATP; p<0.05 for cytosol ATP) (Figure 5A-B).

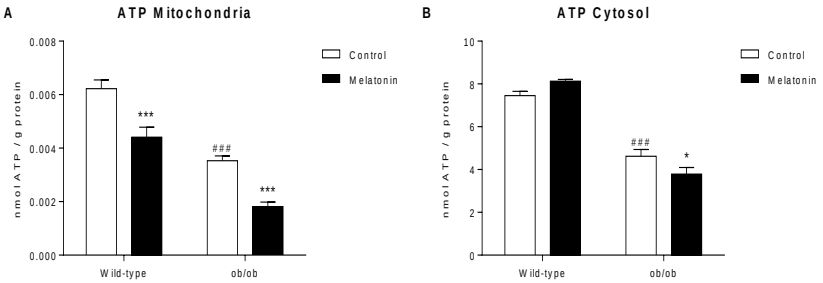

Fig. 5 Effects of melatonin on ATP contents in the brain of wild-type and ob/ob mice.

*Bar charts show the levels of (A) mitochondrial and (B) cytosolic ATP expressed as nmol ATP/g protein. The data are presented as the mean ± standard deviations (SD) 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.*

The levels of mitochondrial uncoupling protein 2 (UCP2) were measured to check if a proton leak occurred in mitochondrial inner membrane which relates to the lower production of ATP. Ob/ob mice did not show variations in UCP2 levels compared with wild-type animals, while melatonin treatment decreased these levels in wild-type mice (p<0.01) and increased then in leptin-deficient animals (p<0.01) (Figure 6).

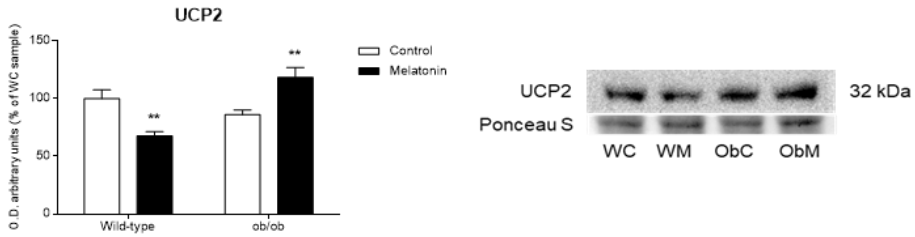

Fig. 6. Effects of melatonin on the expression of UCP2 in the brain of wild-type and ob/ob mice.

*Bar chart shows the semiquantitative optical density (arbitrary units of blots bands) of uncoupling protein 2 (UCP2) from western blots normalized to Ponceau S and expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

3.4. Leptin deficiency-induced obesity alters energy metabolism.

The results have indicated that leptin deficient-induced obesity may alter mitochondrial electron transport chain efficiency by impacting the synthesis of their complexes. Thus, the energy metabolism via glycolysis and the Krebs cycle was examined. This includes to measure the levels of hexokinase II and malate dehydrogenase 2 (MDH2). Our results show that ob/ob mice have lower levels of hexokinase II (p<0.01) and higher levels of MDH2 (p<0.001) than wild-type animals (Figure 7A-B). On the other hand, melatonin treatment decreased hexokinase II levels both in wild-type (p<0.001) and in ob/ob mice (p<0.001) (Figure 7A). Conversely, melatonin elevated MDH2 levels in wild-type animals (p<0.001) and reduced its levels in leptin-deficient mice (p<0.001) (Figure 7B).

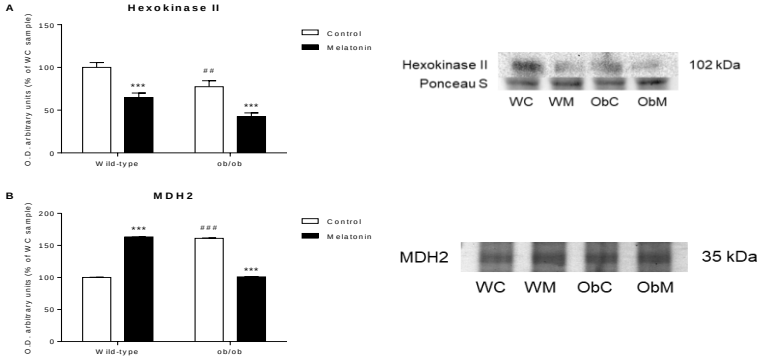

Fig. 7. Effects of melatonin on energy metabolic enzymes in brain of wild-type and ob/ob mice.

*A: Bar chart shows the semiquantitative optical density (arbitrary units of blots bands) of hexokinase II from western blots normalized to Ponceau S and expressed as percent change from the WC sample. B: Bar chart shows the semiquantitative optical density (arbitrary units of blots bands) of malate dehydrogenase 2 or mitochondrial (MDH2) 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 data are expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

3.5. Effects of leptin efficiency associated obesity on the mitochondrial Autophagy.

The following study is to investigate whether this obesity also influences the mitochondrial autophagy. Thus, the expression of Nix and Bcl-2 interacting protein 3 (BNIP3) were measured. Ob/ob animals have lower levels of Nix (p<0.001) and BNIP3 (p<0.001) than that of wild-type mice (Figure 8A-B). Treatment with melatonin reduced Nix levels both in wild-type (p<0.01) and in ob/ob mice (p<0.001), whereas, melatonin treatment only reduces BNIP3 levels in the wild-type mice (p<0.001) but not in ob/ob animals (Figure 8A-B).

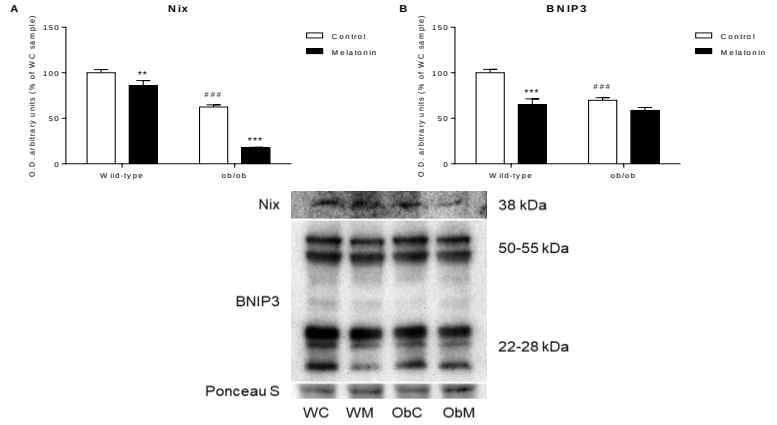

Fig. 8. Effects of melatonin on mitophagy markers in brain of wild-type and ob/ob mice.

*Bar chart shows the semiquantitative optical density (arbitrary units of blots bands) of (A) Nix and (B) Bcl-2 Nineteen-kilodalton Interacting Protein 3 (BNIP3) from western blots normalized to Ponceau S and expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

In addition, Tom20 was also quantified to determine the amounts of mitochondria presented in the different strains of mice. Leptin-deficient mice showed a higher amount of Tom20 than wild-type animals (p<0.05) (Figure 9). On the other hand, melatonin treatment increased the amount of Tom20 in wild-type mice (p<0.001) and decreased its amount in ob/ob animals (p<0.001) (Figure 9).

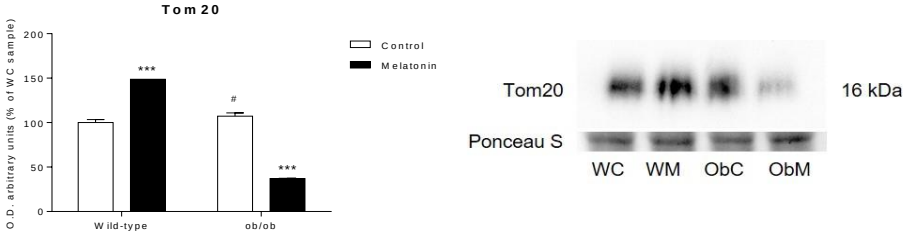

Fig. 9 Effects of melatonin on mitochondrial marker in brain of wild-type and ob/ob mice.

*Bar chart shows the semiquantitative optical density (arbitrary units of blots bands) of Tom20 from western blot normalized to Ponceau S and expressed as percent change from the WC sample. The data are presented as the mean values ± standard deviations (SD) . 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.*

3.6. Effects of Leptin-deficiency and melatonin on the mitochondrial dynamic alterations.

Mitochondrial dynamics and autophagy are related processes. When mitophagy is not activated, it is usually related to mitochondrial fusion process; when it is activated, it is commonly related to mitochondrial fission process.

In this study, mitofusin-2 and dynamin-related protein 1 (DRP1) levels and their ratio was measured. Ob/ob mice had higher levels of Mitofusin-2 ($p<0.05$) and DRP1 ($p<0.001$) than wild-type animals (Figure 10A-B). Melatonin treatment further increased mitofusin-2 levels in wild-type mice ($p<0.05$); on the contrary, it reduced DRP1 levels both in wild-type ($p<0.01$) and ob/ob mice ($p<0.001$) (Figure 10A-B). Regarding mitofusin-2/DRP1 ratio, obesity did not modify this parameter but melatonin treatment increased it both in wild-type ($p<0.05$) and in ob/ob animals ($p<0.001$) (Figure 10C).

These results suggest that leptin deficiency-induced obesity does not modify mitochondrial dynamics but melatonin treatment causes the mitochondria of ob/ob mice to undergo fusion.

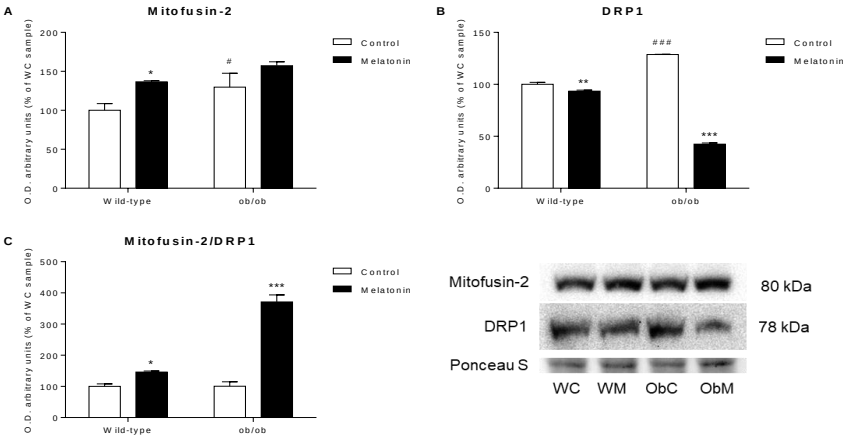

Fig. 10 effects of melatonin on the mitochondrial dynamics in the brain of wild-type and ob/ob mice.

*Bar charts show the semiquantitative optical density (arbitrary units of blots bands) of (A) Mitofusin-2, (B) dynamine-related protein 1 (DRP1) and (C) Mitofusin-2/DRP1 ratio from western blots normalized to Ponceau S and expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

3.7. Effects of melatonin and Leptin deficiency associated obesity on apoptosis.

The primary apoptotic markers, B-cell lymphoma 2 protein (Bcl-2), Bcl-2 associated X (Bax), Bcl-2 associated agonist of cell death (Bad) and p53 upregulated modulator of apoptosis (Puma) were measured. Bcl-2 is an anti-apoptotic marker, while Bax, Bad and Puma are pro-apoptotic markers. The results show that ob/ob mice have similar levels of Bcl-2 and Bax, low level of Bad ($p<0.001$) and high level of Puma compared with wild-type mice (Figure 11A-D). Conversely, melatonin treatment had no significant effect on Bcl-2 but decreased Bax ($p<0.001$) and Bad ($p<0.001$) levels and increased Puma levels in wild-type animals (Figure 11A-D). In the ob/ob mice melatonin reduced Bcl-2 and Puma levels ($p<0.001$), increased Bax levels ($p<0.001$) but had no significant effect on Bad levels (Figure 11A-D).

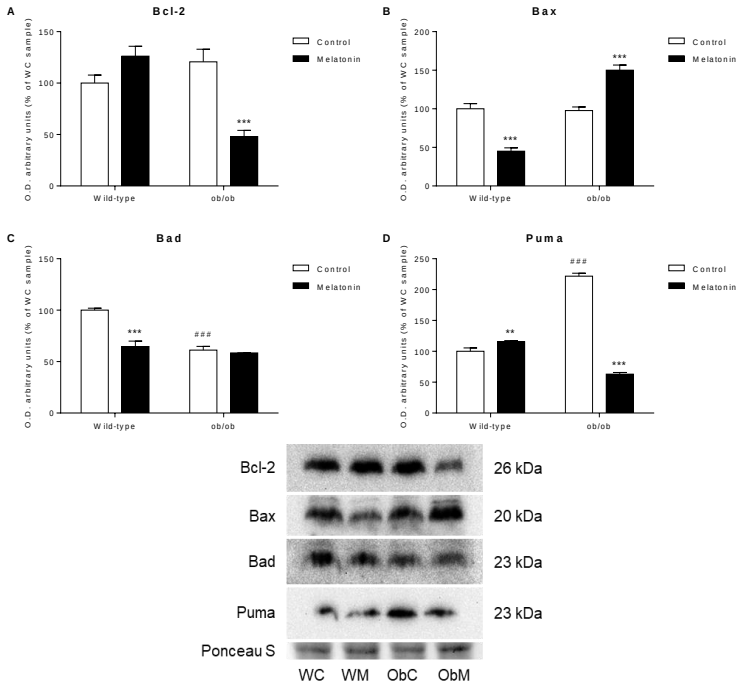

Fig. 11. Effects of melatonin on the apoptotic markers in the brain of wild-type and ob/ob mice.

*Bar charts show the semiquantitative optical density (arbitrary units of blots bands) of (A) Bcl-2, (B) Bax and (C) Bad from western blots normalized to Ponceau S and expressed as percent change from the WC sample. The data are presented as the mean ± standard deviations (SD) 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.*

4. DISCUSSION

In obesity, the production of free radicals and the associated oxidative stress occur preferentially in mitochondria due to excessive fatty acids and glucose supply that leads to overloading the electron transport chain (50). This statement, referred by Vincent and Taylor in 2006, applies to the brain as well. Mitochondria in brain of obese animals showed reduced level of protein subunits of the electron chain complexes, except for complex II, which was greatly increased in ob/ob animals, together with a lower mitochondrial respiration. The main respirasome in the inner mitochondrial membrane is composed of complexes I, III and IV, that improve electron channeling and control ROS production (51). Damage in complex I induces complex II activity, which is increased in several pathologies, and is a major player in the generation of ROS in mitochondria (52). This was observed in obese mice who displayed a decoupled electron transport chain when the respiration was initiated from complex II. These alterations in oxidative phosphorylation caused an abrupt reduction in ATP production, clearly promoting a bioenergetic malfunction elicited by a remodeling in mitochondrial function. The consequences of such a change in an organ such as in the brain is significant because of it is ATP-dependent.

The role of complex II cannot be underestimated , since it is at the crossroads of mitochondrial respiration and tricarboxylic acid cycle which is a central metabolic pathway responsible for anabolic substrates and cell growth processes (53); both of these are exacerbated in obesity. In ob/ob mice, the cycle activation was verified, not only by the increase

in SUC dehydrogenase or complex II, but also for the significant malate dehydrogenase 2 increment.

A dysfunction in processes of energy production is at the center of neurological pathologies (54) and recent studies have linked dysfunction in complex I (55, 56), as well as increases in complex II (57) to the development of depressive disorders and neurodegenerative diseases, such as Alzheimer and Parkinson. Obesity, in light of our findings, is not an exception, since the reduction in neural mitochondrial capacity in ob/ob mice fits perfectly with neurodegenerative biomarkers previously described by our group (19). These mitochondrial alterations and the subsequent neurodegenerative consequences may have hypoxia as a primary inducer. Flow limited breathing together with the collapse of the pharynx have been associated with genetic forms of leptin deficiency and leptin resistance in mice (58, 59). Likewise, pseudohypoxia based on stabilization of hypoxia-inducible factor 1 alpha (HIF-1α) is linked to SUC dehydrogenase dysfunction (52) which is also present in leptin-deficiency obesity. Although the link between leptin resistance, oxidative stress and several pathogenesis have been well established, hormonal treatment, such as amylin and bariatric surgery, have not achieved expected results and it seems lack of accepted medical treatment for leptin resistance (60).

It is striking that in view of such strong established associations, the possible beneficial role of melatonin on leptin-deficiency obesity in brain has not been studied heretofore.

Melatonin has shown its protective capability on mitochondria under several conditions (61, 62), many of them in brain pathologies (63, 64). Likewise, evidence strongly supports that melatonin also improves glucose metabolism (65-67), although its role in this process is still debated (67). Melatonin may have an important dual role in obesity by reducing energy intake and improving mitochondrial capability. Our results support this hypothesis, since melatonin increases expression of complexes I, III and IV. These complexes interact with each other, composing the main respirasome and being the major control system for the production of free radicals in mitochondria (51). The recovery of the complexes III, IV and complex I leads to a reduction in the expression levels of the complex II to its control level under its exacerbated expression caused by obesity. This was also observed under fat biogenesis (68). This reduction in the complex II implies a consequent reduction in the activity of the complex II and, with it, an elevated intracellular SUC (69). SUC dehydrogenase oxidizes SUC to fumarate and transfers electrons from the first one to ubiquinone, re-wiring oxidative phosphorylation and increasing complex II-induced ROS. The recovery of SUC levels with melatonin treatment, observed in our obese animals, leads to a normalization of the Krebs cycle, which was corroborated by malate dehydrogenase which also significantly reduced its levels. By another indirect means, complex II reduction entails a decrement of Reverse Electron Transfer to complex I and, in turn, CI-derived ROS generation (52). Moreover, a reduction in mitochondrial complex II involving SUC recovery seems to be heavily implicated in nutrient storage and energy expenditure as was shown in preadipocytes (70). The differentiation of these cells was predominantly directed to the beige adipocyte, rather than to white adipocyte under suppression of mitochondrial complex II. This study showed a prominent role of UCPs in this process (68). Herein, a significant increase in UCP2 expression was induced by melatonin in obese animals, being coordinated and combined with a measured complex V reduction and a decline in ATP synthesis. The capability of melatonin for enhancing expression of UCP has been previously described under metabolic disturbances; this may be one of the effective strategies developed to recover glucose homeostasis and total metabolic profile (70-72) and, even in melatonin-treated pinealectomized rats (15). Our results expand the knowledge related to the advantageous actions of melatonin through UCP activation; this influences obesity mediated chances at the mitochondrial level in the brain, reducing their ATP production under the starvation signal induced by leptin deficiency of ob/ob animals.

The deficiency of leptin in obese mice induces a starvation signal that has a direct impact on glucose metabolism, especially, at the brain level where glucose is main source of energy. Therefore, this condition has a direct impact, not only at mitochondrial level, but also an anaerobic glycolysis and cytosolic ATP synthesis.

HKII upregulation is considered a major contributor to the elevated glycolysis observed in cancer (73), also known as the Warburg effect. Although this is a process usually limited to cancer cells, obesity, due to its permanent starvation response, could duplicate, at the cytosolic level as in the observed up-regulation in cancer cells. HKII is a major contributor of Warburg effect (73). Thus, HKII regulation would allow to modulate essential strategic cellular processes and, in this study, melatonin treatment induced a significant decrease of this enzyme. Melatonin's effect on glucose metabolism and against the Warburg effect have been recently described and either involves circadian regulation of glucose metabolism (67) or via its glucose metabolism reprogrammation and Warburg reduction (44, 62, 74). Although there is debate regarding the metabolic effects of melatonin, our data strongly support that its effect could be mediated by HKII reduction, which includes either mitoHKII or cytosolic HKII (25). Dissociation mitoHKII is a relevant cellular event usually induced by adverse conditions, such as ischemia or cancer; this can have protective actions into the cell, triggering mitophagy (26) and increasing NADPH levels in the cytosol with measured antioxidant consequences (75). In the subjective situation of our leptin-deficient animals, anchored to a permanent starvation feeling, cytosolic HKII facilitated autophagy during starvation (25), which would aggravate the situation. Our data indicate, conversely, that the reduction in HKII corresponds mainly to mitochondria. The mitophagy reduction, as well as the decrease in total mitochondrial mass, shown by Tom20, seems to support this fact.

In support of this, Bcl-2 proteins have been implicated in mitoHKII dissociation and mitophagy activation (26, 76). This is supported by accumulated evidence which reveals that HKII is not only a molecule involved with glycolysis, but also it has a crucial role in cellular protection by preventing the mitochondrial death pathway (25). Our leptin-deficient animals treated with melatonin showed a significant reduction in Bcl-2 level. Cell death triggered via the mitochondria seems to be promoted; however, scientific evidence that melatonin protects mitochondria against countless adverse events is substantial (34, 62, 77). Therefore, the reduction in Bcl-2 rather seems to provoke a delicate readjustment of pro and anti-apoptotic proteins in the mitochondria to preserve cell survival, for example, reduced ratio of Bax/Bcl-2 with melatonin treatment As seen herein, the reduction in Bcl-2 induces a marked rise in the pro-apoptotic protein Bax, which is the inductor of a major mitochondrial death pathway (78). In turn, activation of Bax induces a pore at the mitochondrial outer membrane thereby releasing, from the intra-membrane space, apoptotic factors (25, 79). The Bax increase does not directly imply apoptosis. Activation of this factor is essential, but not sufficient, for apoptosis triggering, since it is necessary to assess that the most important Bax activator, Puma, which showed a drastic reduction in leptin-deficient animals treated by melatonin. Moreover, Puma has shown to be increased under high glucose treatment while melatonin reduced its expression, alleviating high glucose-induced apoptosis (80). Finally, the significant increase in brain weight observed in ob/ob animals after melatonin treatment is consistent with the anti-apoptotic role of melatonin in brain of these animals.

Melatonin has pleotropic actions in brain mitochondria of leptin-deficient animals. It improves the electron transport chain capacity, suppressing unnecessary complex II activity and, thus, preserving SUC for the Krebs cycle. Likewise, melatonin activates UCPs, which dissipates the extra energy to heat to reduce the obesity. At same time, melatonin reduces anaerobic glycolysis, via HKII reduction to reduce cytosolic ATP production and blockade of mitophagy. Finally, it interrupts apoptosis by directly acting on the pathway activators (Figure 12).

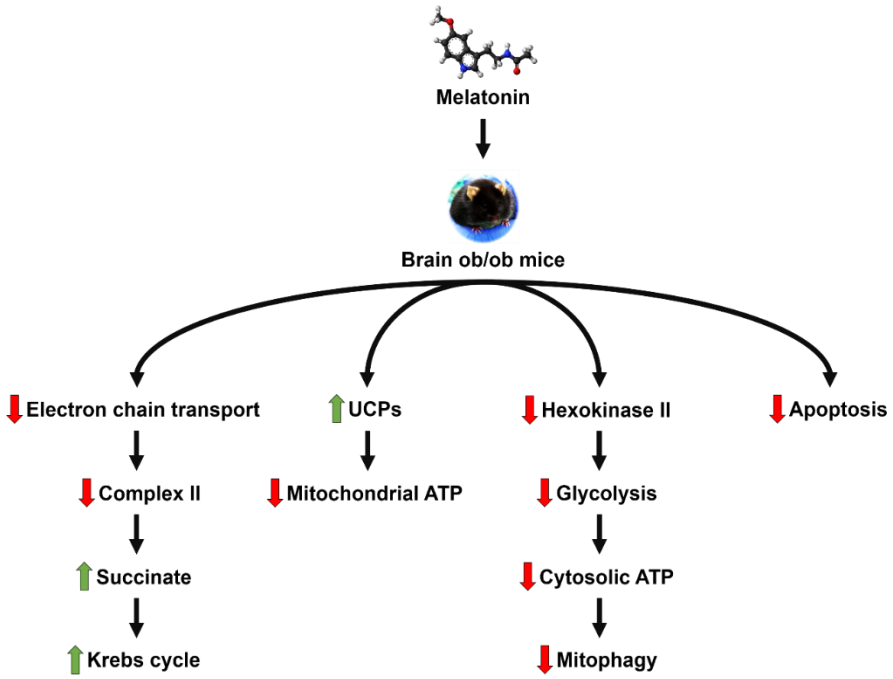

Fig. 12 Global effect of melatonin treatment in the metabolism in the brain of ob/ob mice.

The action of melatonin in this scenario is a continuous balancing act, with clear beneficial actions against cerebral deleterious effects of permanent leptin deficiency. It should be noted that the current leptin-deficient state is a genetic alteration, yet melatonin is an excellent palliative, at least, at the mitochondrial level.

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AUTHORSHIP

A. R-G., B. de L-D. and Y.P. performed the experiments. A. R-G. performed data analysis. A. C-M. and I. V-N. conceptualized and designed the study. B. C., JA. B. and JJ. S. provided suggestions and revised the manuscript. A. R-G. and A. C-M. wrote the manuscript. I. V-N., A. C-M. and RJ. R. supervised and extensively reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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