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Alzheimer's disease and type 2 diabetes-related alterations in brain mitochondria, autophagy and synaptic markers

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ABSTRACT

We aimed to investigate mitochondrial function, biogenesis and autophagy in the brain of type 2 diabetes (T2D) and Alzheimer's disease (AD) mice. Isolated brain mitochondria and homogenates from cerebral cortex and hippocampus of wild-type (WT), triple transgenic AD (3xTg-AD) and T2D mice were used to evaluate mitochondrial functional parameters and protein levels of mitochondrial biogenesis, autophagy and synaptic integrity markers, respectively. A significant decrease in mitochondrial respiration, membrane potential and energy levels was observed in T2D and 3xTg-AD mice. Also, a significant decrease in the levels of autophagy-related protein 7 (ATG7) and glycosylated lysosomal membrane protein 1 (LAMP1) was observed in cerebral cortex and hippocampus of T2D and 3xTg-AD mice. Moreover, both brain regions of 3xTg-AD mice present lower levels of nuclear respiratory factor (NRF) 1 while the levels of NRF2 are lower in both brain regions of T2D and 3xTg-AD mice. A decrease in mitochondrial encoded, nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) was also observed in T2D and 3xTg-AD mice although only statistically significant in T2D cortex. Furthermore, a decrease in the levels of postsynaptic density protein 95 (PSD95) in the cerebral cortex of 3xTg-AD mice and in hippocampus of T2D and 3xTg-AD mice and a decrease in the levels of synaptosomal-associated protein 25 (SNAP 25) in the hippocampus of T2D and 3xTg-AD mice were observed suggesting synaptic integrity loss. These results support the idea that alterations in mitochondrial function, biogenesis and autophagy cause synaptic damage in AD and T2D.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder well recognized by its widespread deleterious effects throughout the body with the brain representing one of its major targets. It is widely accepted that persons suffering from T2D are at increased risk for cognitive decline and the development of Alzheimer's disease (AD) [1], often described as type 3 diabetes [2].

Neurons are metabolic active cells with high energy demands and depend almost exclusively on mitochondria to obtain energy. The maintenance of a healthy mitochondrial pool depends on a delicate equilibrium between several processes including mitochondrial biogenesis and autophagy.

Mitochondrial biogenesis, a cellular process under tight regulation of the nuclear genome, is of extreme significance in keeping a healthy cellular homeostasis [3]. Indeed, in the initial steps of several pathological conditions, alterations of mitochondrial biogenesis and expression of nuclear genes encoding mitochondrial proteins are interpreted as adaptive responses to mitochondrial dysfunction or high energy demands [4,5]. However, there is a lack of information about the possible alterations arising in this process in neurodegenerative diseases, and their role in neuronal injury, although some studies already started to dwell on this subject (for further review please see [6,7]). Evidence from the literature shows a reduction in peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) levels and in mitochondrial number in the hippocampus of AD patients [8,9] and in M17 cells overexpressing mutant APP [10] suggesting impaired mitochondrial biogenesis in AD. Additionally, alterations in mitochondrial biogenesis are also involved in diabetes. It was previously reported that high fat feeding leads to a decline in sirtuin (SIRT) 1 gene expression in the hippocampus of mice suggesting a decrease in mitochondrial biogenesis [11], given that SIRT1 activation is described as being responsible for the deacetylation and activation of PGC-1α, which in turn stimulates mitochondrial biogenesis [12]. By the contrary, a recent

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study from our group showed that in 6-month-old non-obese T2D rats there are no significant alterations in brain mitochondrial biogenesis [13].

Autophagy is a cellular recycling process, accountable for the degradation of intracellular components including proteins and organelles, through lysosomal degradation. Thus, the autophagic process engages a fundamental role as cellular homeostatic and "housekeeping" mechanism [14]. Evidence from the literature shows that autophagy plays a key role in neuronal function being responsible for the removal of damaged proteins and organelles [15]. Although the role of autophagy in central nervous system is still unclear, it is already known that this process is deeply involved in stress responses and cell death pathways [14]. It was previously shown that autophagy is involved in AD-related protein aggregation through an increased accumulation of autophagic vesicles in the cerebral cortex [2,16] and, consequently, an accumulation of amyloid β (Aβ) peptide, since autophagosomes were already demonstrated to be an active compartment for Aβ generation [17], and responsible for the accumulation of dysfunctional mitochondria in neurons due to the inefficient degradation of damaged mitochondria [18,19]. Additionally, the existence of abnormalities in the autophagic process was also described in diabetic patients and cellular and animal models of the disease [20–23]. Despite those studies, the role of autophagy in diabetes remains uncertain.

We have previously shown that T2D and AD mice present a similar profile of brain vascular, mitochondrial and oxidative abnormalities [24,25]. In this line, the current study aimed to demonstrate that brain mitochondrial function, biogenesis and autophagy are impaired in T2D and AD mice, contributing to the loss of synaptic integrity and, probably, synapse loss. Here we evaluated mitochondrial functional parameters in isolated brain mitochondria while brain cortical and hippocampal homogenates were used to evaluate the protein levels of markers of autophagy [mTOR, phosphoinositide 3-kinase (PI3K) p110, beclin, PI3K class III, p62, parkin, B-cell lymphoma 2 (BCl_2) and LAMP1], mitochondrial biogenesis [nuclear respiratory factors 1 and 2 (NRF 1 and NRF 2) and mitochondrial transcription factor A (mTFA), NADH dehydrogenase subunit 1 (ND1), a mitochondrial-encoded complex I subunit, and 70 KDa complex II subunit, a nuclear encoded protein] and synaptic integrity [postsynaptic density protein 95 (PSD95), synaptosomal-associated protein 25 (SNAP 25) and synaptophysin].

2. Material and methods

2.1. Animals

4-Month-old male wild type (WT) mice and triple transgenic mice for AD (3xTg-AD) were housed in our animal colony (Animal Facility, Faculty of Medicine/Center for Neuroscience and Cell Biology, University of Coimbra). WT mice were randomly divided into two groups: 1) control group and 2) sucrose-treated WT mice with free access to 20% sucrose solution during 7 months to induce a diabetic phenotype. Mice were maintained under controlled light (12 h day/night cycle) and humidity with free access (except in the fasting period) to water (WT and 3xTg-AD mice at basal conditions) or 20% sucrose solution (T2D) and powdered rodent chow (URF1; Charles River). Adhering to procedures approved by the Federation of Laboratory Animal Science Associations (FELASA), mice (11-month-old) were sacrificed by cervical dislocation and decapitation at the end of treatment period.

2.2. Determination of biochemical parameters

Blood glucose levels were determined by a glucose oxidase reaction, using a commercial glucometer (Glucometer-Elite Bayer, Portugal) and compatible reactive tests (Ascencia Elite Bayer, Portugal). Blood insulin levels were determined using a commercial mouse insulin kit (Mercodia, Arium Barbosa Portugal). Blood glycated hemoglobin A1C (HbA1C) levels were determined using a commercial DCA Vantage™ analyzer (Siemens HealthCare Diagnostics, Portugal) and compatible HbA_{1C} reactive tests (Siemens HealthCare Diagnostics, Portugal).

2.3. Mitochondrial fraction isolation

Brain mitochondria were isolated from mice by the method described by Carvalho and co-workers [24], adding 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, the mouse was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized at 4 °C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, pH 7.4) containing 1.5 mg of bacterial protease type VIII. Single brain homogenates were brought to 20 ml and then centrifuged at 2500 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10,000 rpm for 10 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended again in 10 ml of medium and centrifuged at 10,000 rpm for 5 min. The pellet was resuspended in 10 ml of washing medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4) and centrifuged at 10,000 rpm for 5 min. The final mitochondrial pellet was resuspended in 100 μl of the washing medium. Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin (BSA) [26].

2.4. Mitochondrial respiration measurements

Oxygen consumption of brain mitochondria was registered polarographically with a Clark oxygen electrode [27] connected to a suitable recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of the standard medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 10 μM EGTA, pH 7.4) with 0.5 mg of protein. The respiratory state 2 of mitochondrial respiration was initiated with 5 mM succinate (mitochondrial energization through complex II) in the presence of 2 μM rotenone. The respiratory control ratio (RCR) is the ratio between respiratory states 3 (consumption of oxygen in the presence of succinate and 155 nmol ADP/mg protein) and 4 (consumption of oxygen after ADP has been consumed).

2.5. Mitochondrial membrane potential (ΔΨm) measurements

ΔΨm was monitored by evaluating the transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared according to Kamo et al. [28] using an Ag/AgCl-saturated electrode (Tacussel, model MI 402) as reference. TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Kipps and Zonen recorder. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. Reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard medium containing 3 μM TPP^+. This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria [29]. The ΔΨm was estimated by the equation: $\Delta\Psi m\ (mV) = 59 \log(v/V) - 59 \log(10^{\Delta E/59} - 1)$ as indicated by Kamo et al. [28] and Muratsugu et al. [30]. v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation, and that the law of mass conservation is applicable. A matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes, because the purpose of the

experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on ΔΨm values. However, the overestimation is only significant at ΔΨm values below 90 mV, therefore, far from our measurements. Mitochondria (0.5 mg/ml) were energized with 5 mM succinate in the presence of 2 μM rotenone. After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), ΔΨm fluctuations were recorded.

2.6. Determination of adenine nucleotide levels

At the end of each ΔΨm measurement, 250 μl of each sample was promptly centrifuged at 14,000 rpm (Eppendorf centrifuge 5415C) for 2 min with 250 μl of 0.3 M perchloric acid ($HClO_4$). The supernatants were neutralized with 10 M KOH in 5 M Tris and again centrifuged at 14,000 rpm for 2 min. The resulting supernatants were assayed for adenine nucleotide by separation in a reverse-phase high performance liquid chromatography (HPLC). The HPLC apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5 μm) from Merck. An isocratic elution with 100 mM phosphate buffer (KH_2PO_4; pH 6.5) and 1.2% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 5 min. Adenine nucleotides were identified by their chromatographic behavior (retention time, absorption spectra and correlation with standards).

2.7. Protein extraction for Western blot

Cerebral cortices and hippocampi were homogenized in buffer containing 50 mM trizma hydrochloride (Tris–HCl), 150 mM sodium chloride (NaCl), 1% NP-40, 1% sodium deoxycholate (DOC) and 0.1% sodium dodecyl sulfate (SDS) (pH 7.4), protease inhibitors (commercial protease inhibitor cocktail from Roche), phosphatase inhibitors (commercial phosphatase inhibitor cocktail from Roche), 0.1 M phenylmethanesulfonyl fluoride (PMSF) (Sigma), 0.2 M dithiothreitol (DTT) (Sigma), frozen three times in liquid nitrogen and centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415C) for 10 min. The supernatants represent the cytosolic fractions and the resulting pellets the membrane fractions. The amount of protein content in the samples was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce).

Table 1
List of primary antibodies used in Western blot analyses.

Protein	Reference	Dilution
ATG7	Cell Signaling catalog #2631	1:1000
β-Actin	Sigma Aldrich catalog #A5441	1:5000
Bcl-2 (50E3)	Cell Signaling catalog #2870	1:1000
Beclin	BD Bioscience catalog #612113	1:1000
LAMP 1 (C54H11)	Cell Signaling catalog #3243	1:1000
LC3B	Sigma Aldrich catalog # L7543	1:1000
Phospho-mTOR (Ser2448)	Cell Signaling catalog #2971	1:1000
mtTFA (A-17)	Santa Cruz Biotechnology catalog # sc-23588	1:1000
mTOR (L27D4)	Cell Signaling catalog #4517	1:1000
ND1 (C-18)	Santa Cruz Biotechnology catalog # sc-20493	1:500
NRF-1 (H-300)	Santa Cruz Biotechnology catalog # sc-33771	1:1000
Nrf2	Abcam catalog # ab31163	1:1000
Anti-p62/SQSTM1	Sigma Aldrich catalog #p0067	1:1000
Anti-Parkin [PRK8]	Abcam catalog #ab77924	1:1000
PI3 Kinase Class III (D9A5)	Cell Signaling catalog #4263	1:1000
PI3 Kinase p110α (C73F8)	Cell Signaling catalog #4249	1:1000
PSD95 (D27E11)	Cell Signaling catalog #3450	1:1000
mtTFA (A-17)	Santa Cruz Biotechnology catalog # sc-23588	1:1000
SNAP 25 (SP12)	Sigma Aldrich catalog #S5187	1:1000
Synaptophysin	Sigma Aldrich catalog #S5768	1:1000

2.8. Western blot

The samples were resolved by electrophoresis in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS) for 1 h at room temperature. The blots were subsequently incubated with specific primary antibodies (Table 1), overnight at 4 °C, with gentle agitation. Blots were washed three times (3 × 10 min), with TBS containing 0.1% Tween (TBS-T) and then incubated with secondary antibodies for 2 h at room temperature with gentle agitation. After three washes with TBS-T specific bands of immunoreactive proteins were visualized after membrane incubation with enhanced chemifluorescence (ECF) for 5 min in a VersaDoc Imaging System (Bio-Rad), and the density of protein bands was calculated using the Quantity One Program (Bio-Rad).

2.9. Statistical analysis

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the one-way ANOVA followed by the post-hoc Tukey's test.

3. Results

3.1. Mice characterization

Sucrose-treated mice presented a significant increase in body weight, and a decrease in brain weight when compared with WT mice. Moreover, sucrose intake promoted an increase in blood glucose, HbA_{1C} and insulin levels (Table 2), when compared with the respective WT control mice. The alterations induced by sucrose intake are phenotypic characteristics of T2D. 3xTg-AD mice presented a significant decrease in body and brain weight (Table 2). Interestingly, 3xTg-AD mice also showed increased HbA_{1C} and postprandial glucose levels when compared with WT control mice (Table 2).

3.2. T2D and 3xTg-AD mice showed impaired mitochondrial function

Mitochondria from 3xTg-AD and T2D mice presented a similar decrease in RCR, ΔΨm and ATP/ADP ratio when compared with mitochondria isolated from WT control mice (Table 3).

3.3. Insulin-dependent regulation of autophagy is not significantly altered in T2D and 3xTg-AD mice

The mammalian target of rapamycin (mTOR) complex has a key role in insulin-dependent regulation of autophagy. No significant alterations in the levels of phosphorylated mTOR (p-mTOR) were observed (Fig. 1B

Table 2
Animal characterization.
Data shown represent mean ± SEM from 6–8 animals. Statistical significance: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ when compared with wt control animals. HbA_{1C} — Blood glycated hemoglobin A1C.

	WT control	WT sucrose	3xTg-AD
Body weight (g)	34.72 ± 1.08	43.41 ± 1.24***	30.74 ± 0.70**
Brain weight (g)	1.06 ± 0.07	0.75 ± 0.08*	0.65 ± 0.07**
Occasional glycemia (mg/dl)	122.40 ± 7.90	144.00 ± 5.58*	119.70 ± 7.62
Insulin levels (μg/l)	1.22 ± 0.19	4.73 ± 0.88***	0.97 ± 0.18
HbA_{1C} %	3.42 ± 0.09	3.81 ± 0.07*	3.93 ± 0.10*

Table 3
Effects of T2D and AD on mitochondrial respiratory control ratio (RCR), membrane potential (ΔΨm) and energy levels.

	WT	T2D	3xTg-AD
RCR	3.36 ± 0.20	2.82 ± 0.08*	2.80 ± 0.12*
ΔΨm (-mV)	223.10 ± 3.98	201.50 ± 2.86***	198.20 ± 1.56***
ATP/ADP	9.19 ± 0.96	4.03 ± 1.20*	4.39 ± 1.38**

These mitochondrial parameters were evaluated in freshly isolated brain mitochondrial fractions (0.5 mg) in 1 ml of the reaction medium supplemented with 3 μM TPP^+ and energized with 5 mM succinate in the presence of 2 μM rotenone. Adenine nucleotide levels (ATP and ADP) were determined by HPLC, as described in Materials and methods section. Data shown represent mean ± SEM from 6–8 independent experiments. Statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001 when compared with WT control animals; $^{\$}$p < 0.05; $^{\$\$}$p < 0.01 when compared with 3xTg-AD animals.

and C). Accordingly, the levels of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (p110), a kinase responsible for the phosphorylation of mTOR, is not significantly changed in both cerebral cortex and hippocampus of T2D and 3xTg-AD mice (Fig. 1D and E).

3.4. The nucleation phase of autophagy is altered in T2D and 3xTg-AD mice

Autophagy is a highly regulated process that can also be activated by an insulin-independent pathway. The autophagy nucleation process occurs through the beclin/parkin/PI3K class III complex. A slight increase in beclin levels, a protein involved in the nucleation phase of autophagy (Fig. 2B and C) in both cerebral cortex and hippocampus was observed in T2D and 3xTg-AD mice. A slight decrease in B-cell lymphoma 2 (BCL_2) levels (Fig. 2F and G), a beclin repressor, in the hippocampus of T2D and 3xTg-AD mice was also observed. Furthermore, an increase in PI3K class III kinase, an activator of beclin-induced nucleation, was observed in the cerebral cortex and hippocampus of both groups of mice although only statistically significant in the cerebral cortex of 3xTg-AD (Fig. 2D and E). Also, the cerebral cortex and hippocampus of 3xTg-AD mice presented a significant increase in parkin levels (Fig. 2H and I).

3.5. The elongation phase of autophagy is disturbed in T2D and 3xTg-AD mice

Autophagy-related genes (ATG) are involved in the control of autophagosome formation, cargo gathering and trafficking to the lysosomal compartment [31]. Therefore, alterations in proteins involved in these mechanisms can compromise the clearance of damage organelles and aggregated proteins. A significant decrease in ATG7 levels in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice (Fig. 3D and E) was observed, suggesting a disruption in the elongation phase of the autophagosome formation. A slight decrease in LC3-II protein levels was also observed in the cerebral cortex of T2D and 3xTg-AD mice (Fig. 3B). No significant alterations were observed on p62 levels (Fig. 3F and G).

3.6. Decreased lysosomal membrane stabilization occurs in T2D and AD mice

The lysosomal-associated membrane protein 1 (LAMP1) is a marker of lysosome mass [32]. No significant alterations were observed in the levels of LAMP1 (Fig. 4F and G). However, a decrease in the levels of glycosylated LAMP1 was observed in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice although only statistically significant in the last group of animals (Fig. 4B and C). Lower LAMP1 glycosylation suggest decreased lysosomal membrane stabilization.

3.7. A decrease in mitochondrial biogenesis is observed in both T2D and 3xTg-AD mice

Since the balance between mitochondrial biogenesis and turnover is essential to maintain a healthy mitochondrial pool, we also evaluated the levels of three key proteins involved in mitochondrial biogenesis, NRF1, NRF2 and mTFA. A decrease in the levels of NRF1 was observed in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice, although only statistically significant in the cerebral cortex of 3xTg-AD mice (Fig. 5D and E). Also, a decrease in NRF2 levels was observed in T2D and 3xTg-AD mice, this decrease being statistically significant in the hippocampus (Fig. 5F and G). An increase in mTFA levels was also observed although only statistically significant in the cerebral cortex of T2D mice (Fig. 5A and B), suggesting a compensatory response to mitochondrial damage. The levels of the mitochondrial encoded ND1 subunit were found to be decreased (Fig. 6B and C), particularly in the cerebral cortex of T2D mice while no alterations were observed in the nuclear-encoded complex II subunit (Fig. 6D and E).

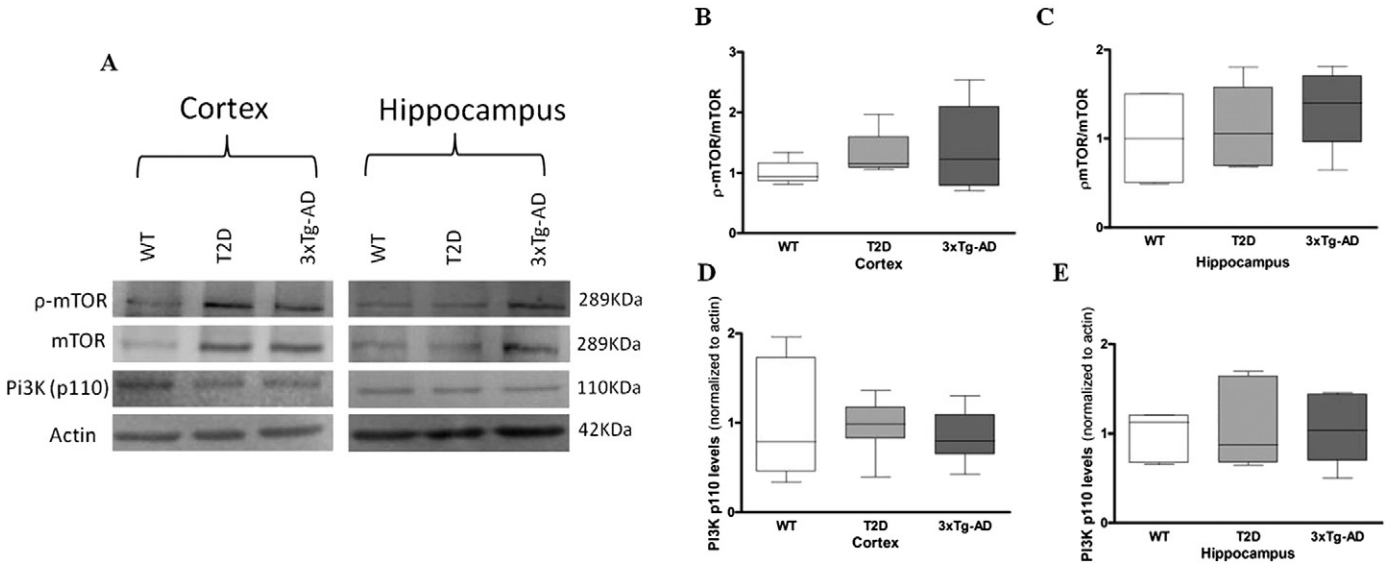

Fig. 1. T2D and AD effects in PI3K (p110)/mTOR-dependent autophagy inhibition. Western blot representative images (A), cortex (B) and hippocampus (C) pmTOR/mTOR and cortex (D) and hippocampus (E) PI3K (p110) protein levels. Data shown represent mean ± SEM from 5–7 animals.

3.8. A loss of synaptic integrity occurs in T2D and AD mice

Since brain cells, particularly neurons, are highly reliant on mitochondria, alterations in mitochondrial homeostasis will have a profound impact on cells' integrity and function. In order to evaluate synaptic integrity, we measured the levels of the presynaptic proteins, SNAP 25 and synaptophysin, and the post-synaptic protein PSD95. A decrease in SNAP 25 was observed in both cerebral cortex and hippocampus of T2D and 3xTg-AD mice reaching statistical significance only in the hippocampus (Fig. 7F and G). Concerning synaptophysin a slight decrease was observed particularly in the hippocampus of T2D and 3xTg-AD mice (Fig. 7D and E). Additionally, we also observed a significant decrease in PSD95 levels in the cerebral cortex and hippocampus of 3xTg-AD mice and hippocampus of T2D mice (Fig. 7B and C).

4. Discussion

This study shows altered autophagy markers in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice, particularly a decrease in ATG7 and glycosylated LAMP1 protein levels, which suggest a defective elongation phase of the autophagosome formation and a destabilization of the lysosomal membranes, respectively. Also, a decrease in NRF1 and NRF2 levels occur in T2D and 3xTg-AD brain cortex and hippocampus, respectively, suggesting a decrease in mitochondrial biogenesis, which may underlie the decrease in ND1 levels, a mitochondrial DNA encoded protein. The imbalance observed in mitochondrial biogenesis and autophagy could contribute to the impairment of mitochondrial function in both T2D and 3xTg-AD mice. Because neuronal cells are highly dependent on a healthy pool of mitochondria, alterations in these organelles will affect neurons structure and function. In fact, a significant decrease in SNAP25 and PSD95 protein levels was observed in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice suggesting a loss of synaptic integrity.

Sucrose intake promoted a significant increase in occasional blood glucose, HbA_{1C}, and insulin levels and body weight (Table 2), which are phenotypic characteristics of T2D [24,25]. Furthermore, 3xTg-AD animals presented a decrease in body and brain weight (Table 2) features also observed in AD patients [33–35].

Mitochondria isolated from the brains of T2D and 3xTg-AD animals presented an impairment of the respiratory chain and oxidative phosphorylation system (Table 3). Together those alterations can be responsible for the lower levels of ATP observed in this study (Table 3). These results are in agreement with previous studies from our laboratory showing that T2D and 3xTg-AD mice present significantly decreased activities of the mitochondrial respiratory enzyme complexes in brain vessels and synaptosomes as well as brain mitochondrial oxidative and ultrastructural alterations [24,25] supporting the idea that mitochondrial dysfunction is a common denominator between T2D and AD.

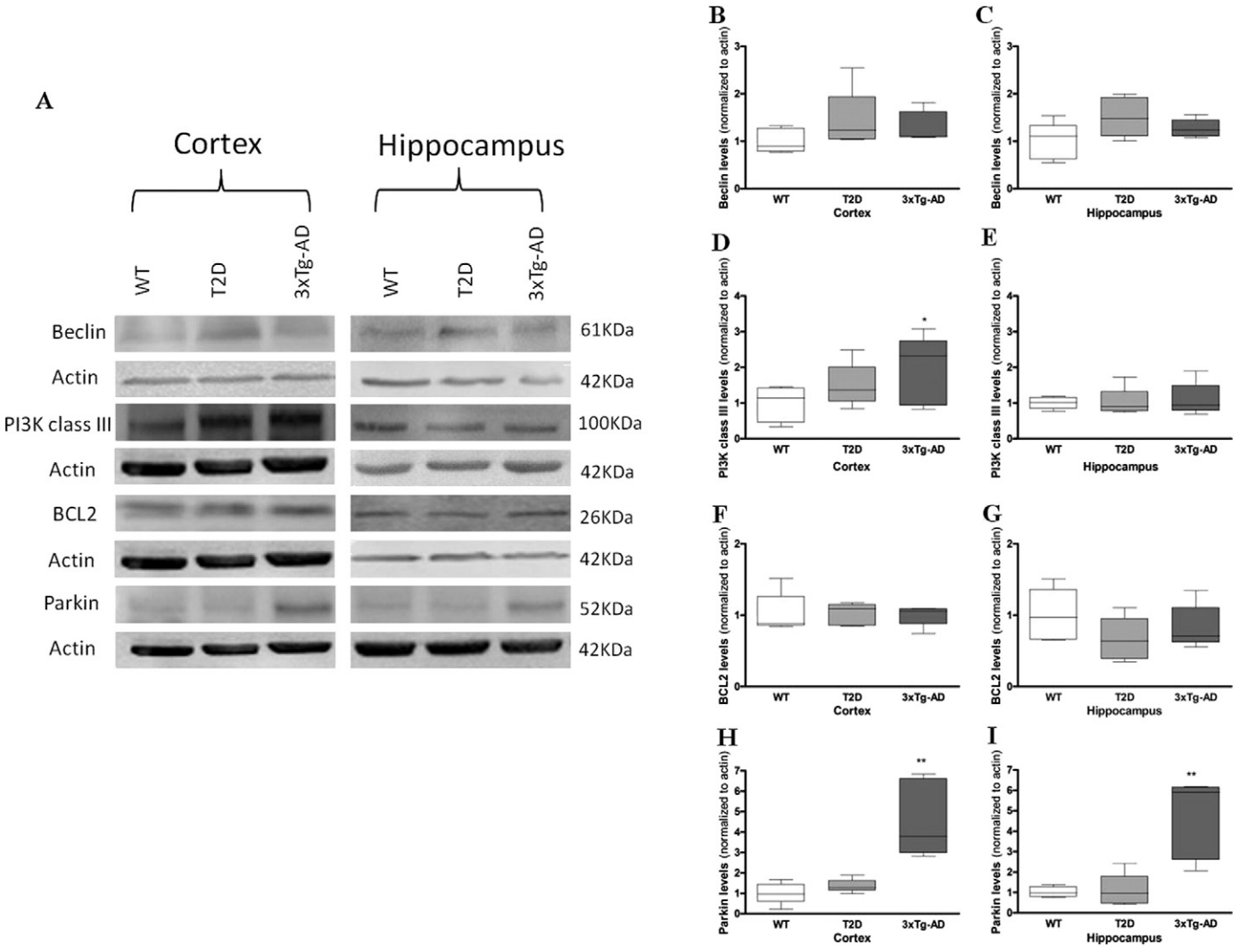

Fig. 2. T2D and AD effects in autophagy nucleation phase. Western blot representative images (A), cortex (B) and hippocampus (C) beclin, cortex (D) and hippocampus (E) PI3K class III, cortex (F) and hippocampus (G) BCL2 and cortex (H) and hippocampus (I) parkin protein levels. Data shown represent mean ± SEM from 5–7 animals.

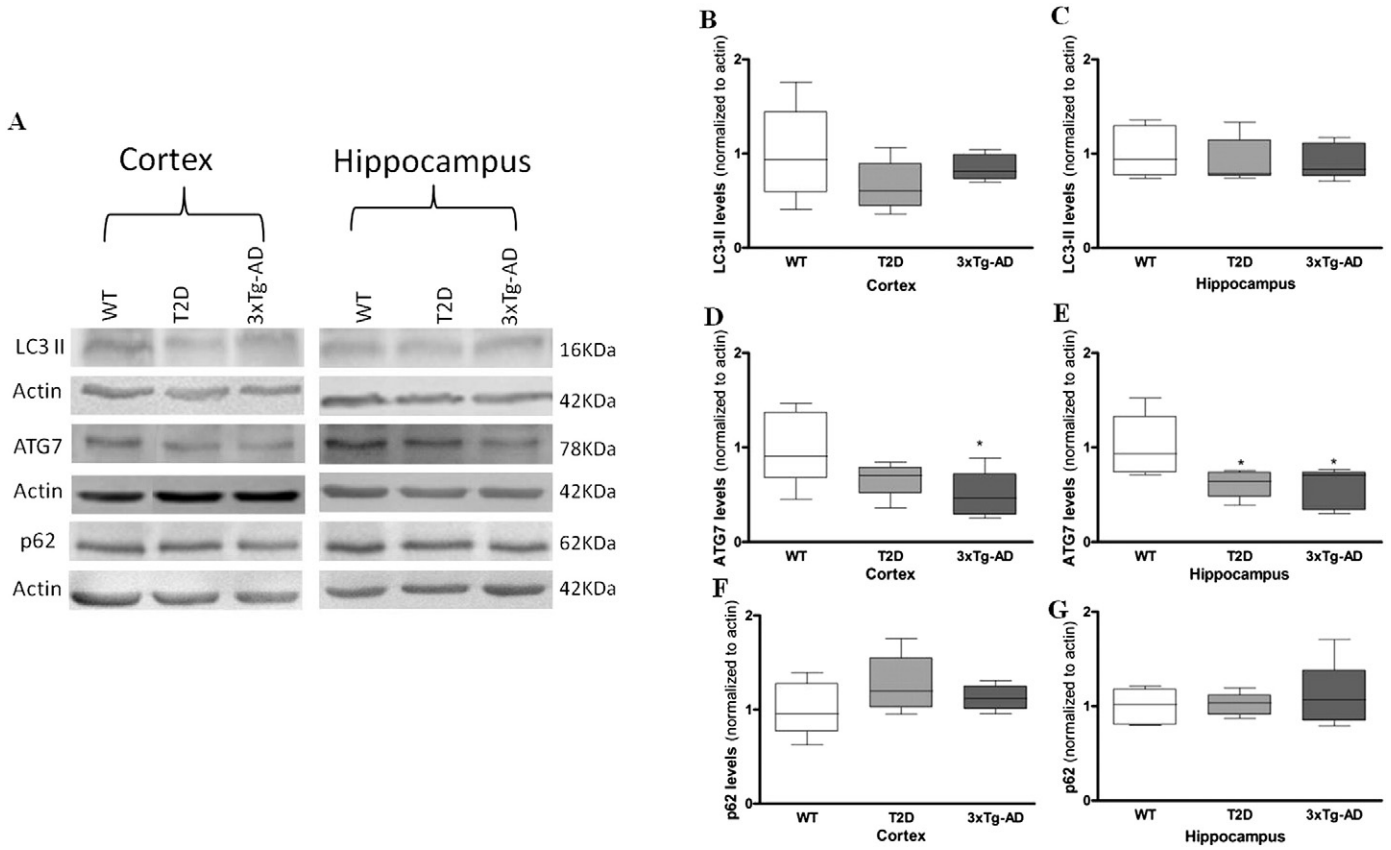

Fig. 3. T2D and AD effects in autophagy elongation phase. Western blot representative images (A), cortex (B) and hippocampus (C) LC3-II, cortex (D) and hippocampus (E) ATG7 and cortex (F) and hippocampus (G) p62 protein levels. Data shown represent mean ± SEM from 5–7 animals.

Insulin and insulin-like growth factors regulate mTOR through the class I PI3K [36]. No significant alterations in PI3K (p110) (Fig. 1D and E) and phosphorylated (activated) mTOR (p-mTOR) (Fig. 1B and C) levels were observed. The role of mTOR in several disorders appears to be conflicting. Indeed, down-regulation of mTOR signaling was reported in the brains of APP/PS1 mutant transgenic mice [37] although in 7PA2 cells overexpressing mutant APP and in brains of PDAPP mice [also known as hAPP(J20)], another AD transgenic mouse, mTOR signaling was shown to be up-regulated [38,39]. A previous study from our laboratory showed that 6-month-old Goto-Kakizaki rats, a model of non-obese T2D, presented an increase in the levels of p-mTOR in the cerebral cortex [13] however, STZ-induced type 1 diabetic rats did not present any alterations in p-mTOR, suggesting that different types and/or duration of diabetes may be characterized by different patterns of mTOR activation. Additionally, studies in lean and obese Zucker rats skeletal muscle also showed that the levels of p-mTOR are not altered [40] while in db/db mice hearts p-mTOR seems to be increased [41] revealing the existence of tissue specificities. Also, it is known that mTOR activation can occur through the phosphorylation of one of three residues and only S2448 (the most common analyzed residue) was evaluated in our study.

Concerning the nucleation phase of autophagy, only a slight increase in the levels of beclin and PI3K class III and a decrease in BCL_2 was observed, particularly in cerebral cortex (Fig. 2B, D and F), although previous studies have shown that the levels of beclin, PI3K class III and BCL_2 are decreased in AD and T2D brains [42–45]. Under physiological conditions BCL_2 inhibits autophagy by sequestering beclin and avoiding its interaction with PI3K class III, which is required for the initiation of autophagy nucleation [44]. However, we must be aware that different models of disease may contribute to distinct observations. Moreover, parkin seems to be implicated in a different pathway involved in the stimulation of autophagic clearance of cellular waste [46]. A statistical significant increase in parkin protein levels in both cerebral cortex and hippocampus of 3xTg-AD mice (Fig. 2H and I) was observed, which is in agreement with the study of Lonskaya and coworkers [47] showing a significant increase in total parkin levels (130%, $p < 0.05$) in human postmortem AD brains. The same authors suggested that an increase in parkin insolubility occur in AD brains that may be related with a decrease in parkin phosphorylation affecting the interaction of parkin with beclin-1 [47].

After nucleation, the autophagosome suffers an elongation phase, a process that involves several proteins such as LC3-II, p62 and ATG7. Although no significant alterations were observed in p62 levels (Fig. 3F and G), the levels of ATG7, an activating enzyme, are significantly decreased in the cerebral cortex and hippocampus of 3xTg-AD and T2D mice (Fig. 3D and E). Furthermore, a slight decrease in LC3-II levels in the cerebral cortex of T2D and 3xTg-AD mice occurred (Fig. 3B). Our results are in agreement with studies performed by Yang and coworkers [48] where both genetic (ob/ob mice) or dietary (through high fat diet) models of T2D presented decreased levels of hepatic ATG7. Indeed, it has been described that a decrease in ATG7 levels leads to a defective evolution of the autophagic process [48] and autophagy can be restored by increasing the expression of ATG7 alone [49]. Furthermore, animals lacking ATG7 protein exhibit motor and behavioral deficits and disruption in intracellular transport and secretion of Aβ, playing a key role in Aβ plaque formation, which emphasizes the importance of a constitutive level of autophagosome formation for neuronal survival even in the absence of any disease factor that could elicit an autophagic response [50–53]. Accordingly, we previously showed that 11-month-old T2D and 3xTg-AD mice present pronounced behavioral deficits [54], alterations that may also be related with ATG7 protein deficits.

Also, a significant decrease in glycosylated LAMP1 levels occurred in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice (Fig. 4B and C), a modification necessary for LAMP1 stabilization in lysosome

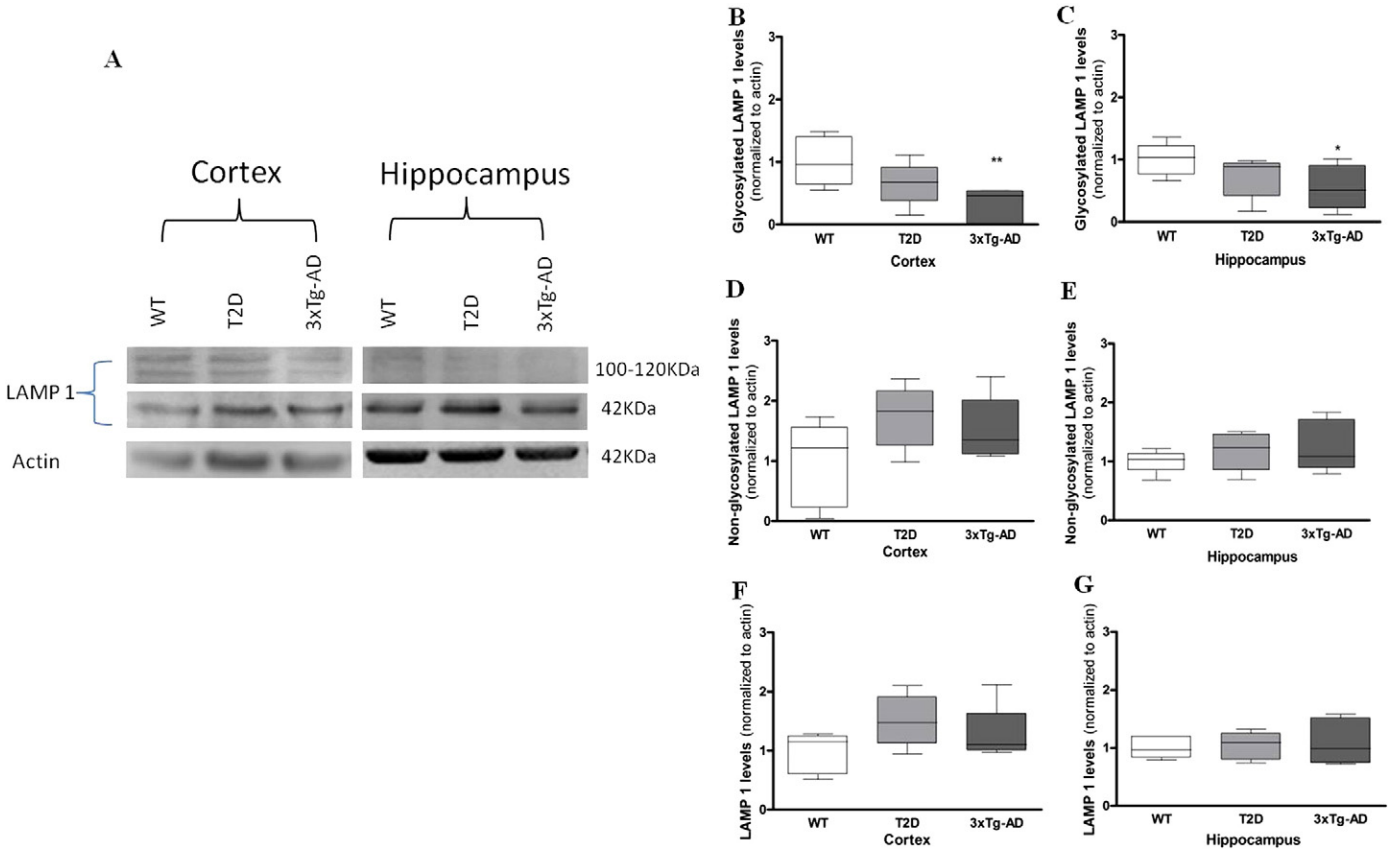

Fig. 4. T2D and AD effects in autophagy maturation phase. Western blot representative images (A), cortex (B) and hippocampus (C) glycosylated LAMP-1, cortex (D) and hippocampus (E) non-glycosylated LAMP-1 and cortex (F) and hippocampus (G) total LAMP-1 protein levels. Data shown represent mean ± SEM from 5–7 animals.

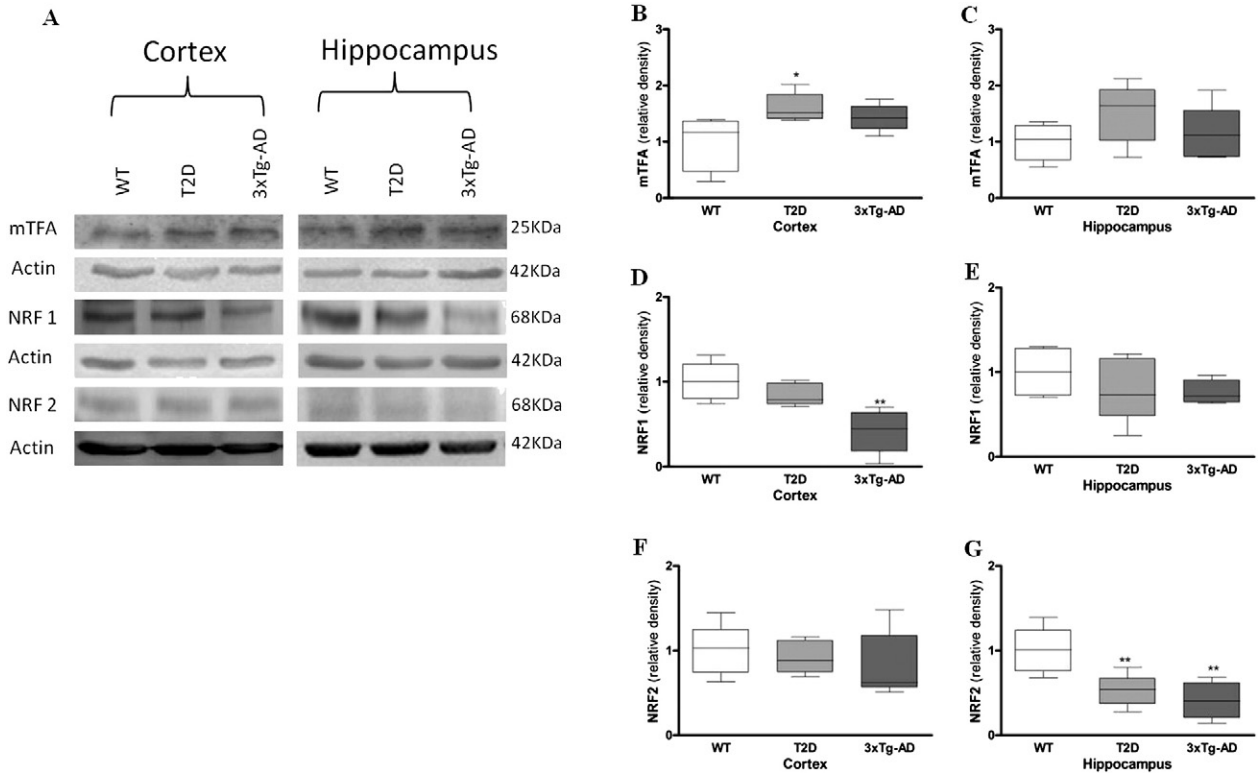

Fig. 5. T2D and AD effects in mitochondrial biogenesis regulation factors. Western blot representative images (A), cortex (B) and hippocampus (C) mTFA, cortex (D) and hippocampus (E) NRF1 and cortex (F) and hippocampus (G) NRF2 protein levels. Data shown represent mean ± SEM from 5–7 animals.

membranes, presumably protecting them against proteolytic degradation [55]. The use of LAMP1 as a marker of lysosome mass is often reported [32] and an increase in LAMP1 levels in AD brains was already showed [56] however, to our knowledge, there are no studies reporting the levels of lysosomal stabilization. This alteration may contribute to the accumulation of autophagosomes, a situation already described in AD patients and animal models [57,58].

The impairment of autophagy is expected to cause the accumulation of toxic proteins such as Aβ and damaged organelles, particularly mitochondria. In fact, a previous study from our laboratory demonstrated that T2D mice brains present an accumulation of Aβ similar to that observed in 3xTg-AD [24,25]. Son and coworkers also showed that the increase in Aβ production in insulin-resistance conditions resulted from an accumulation of autophagosomes due to alterations in autophagic flux resulting from the inhibition of mTOR [2,22].

Besides the alterations observed in the autophagic process, an impairment of mitochondrial biogenesis in both T2D and 3xTg-AD mice (Fig. 5) also seems to occur. It is known that a delicate balance between mitochondrial biogenesis and turnover is essential for the normal function of cells [59]. Since cells are not able to produce mitochondria "de novo", they rely on a transcriptional system of mitochondrial biogenesis that is highly regulated by nuclear regulatory proteins, mainly NRF1, NRF2 and mTFA [60]. NRF1 is a transcriptional activator of nuclear genes that encode a number of mitochondrial respiratory enzymes, including subunits of the five respiratory chain complexes [61]. Likewise, NRF1 is also necessary for the activation of mTFA [62] rendering it essential for mitochondrial biogenesis [61], once it is required for mtDNA transcription and replication [63]. A decrease in NRF1 levels was observed in both T2D and AD, although only statistically significant in 3xTg-AD mice cerebral cortex (Fig. 5D and E). Previous studies showed significant alterations of NRF1 levels in skeletal muscle of GK rats [64] or testis of a high-energy-diet induced pre-diabetic rat model [65]. Also, in retina from streptozotocin-induced type 1 diabetic rats, maintained in poor or good glycemic control, no alterations were observed in NRF1 levels [66] revealing consistent results in this protein levels in diabetic conditions. Pedrós and coworkers observed significant reductions in NRF1 transcripts in APP/PS1 animals, when compared to control animals [67]. NRF2 is also highly involved in mitochondrial biogenesis and antioxidant defense factors expression. In the present study we observed a significant decrease in NRF2 levels in hippocampus of both T2D and 3xTg-AD mice (Fig. 5G). These results are in accordance with the decrease in antioxidant defenses in the brains of T2D and 3xTg-AD mice, as previously reported [24]. It was also observed a decrease in NRF2 transcripts in hippocampi of 3-month-old APP/PS1 animals [67] and presenilin 1/2 knockout cells [68] as well as in the hearts of STZ-induced type 1 diabetic mice and diabetic patients [69]. Furthermore, NRF2 is associated with the transcriptional activity of all ten subunits of cytochrome c oxidase [70], which is in accordance with a previous study demonstrating a decreased activity of cytochrome c oxidase in synaptosomes from T2D and 3xTg-AD mice [25]. Besides NRF1, also NFR2 is involved in the activation of mTFA [62,63]. Surprisingly, we observed an increase in mTFA levels (Fig. 5B and C), particularly in the cerebral cortex of T2D mice. Santos and Kowluru [71] reported that although diabetic rats present an increase in mTFA levels, there is a defective membrane transport of mTFA into the mitochondria, and consequently a decrease in mTFA–mtDNA binding, which results in subnormal mitochondria transcription [71]. We believe that a similar mechanism may occur in the brains of T2D and 3xTg-AD mice since we observed a decrease in ND1 protein levels, particularly in the cerebral cortex of T2D mice (Fig. 6B and C), supporting the idea that under our experimental conditions mTFA could not be activated. ND1 is a mitochondrial encoded protein of mitochondrial respiratory chain and its decreased levels affect mitochondrial function (Table 2), as previously reported [24,25]. Although the decrease in ND1 levels is not so pronounced compared with mitochondrial dysfunction, we must be aware that ND1 protein function may be altered under diseased conditions.

Because mitochondria are essential organelles for neuronal cells, we evaluated synaptic integrity in the cerebral cortex and hippocampus of T2D and 3xTg-AD mice. A decrease in synaptic integrity was described to occur in rodent diabetic brains [72,73]. Oddo and colleagues also reported the existence of synaptic dysfunction in the 3xTg-AD mice including synaptic transmission and long-term potentiation (LTP) deficits [74]. Although the mechanism of synapse loss in AD remains uncertain, several studies reported a loss of synaptic integrity in AD terminals of human brains [75]. We observed a decrease in PSD-95 levels in cerebral cortex and hippocampus of T2D and 3xTg-AD mice, although only statistically significant in the hippocampus (Fig. 7B and C). PSD-95 supports synapse maturation wielding a major influence on synaptic strength and plasticity [76]. On the other hand, only a non-significant

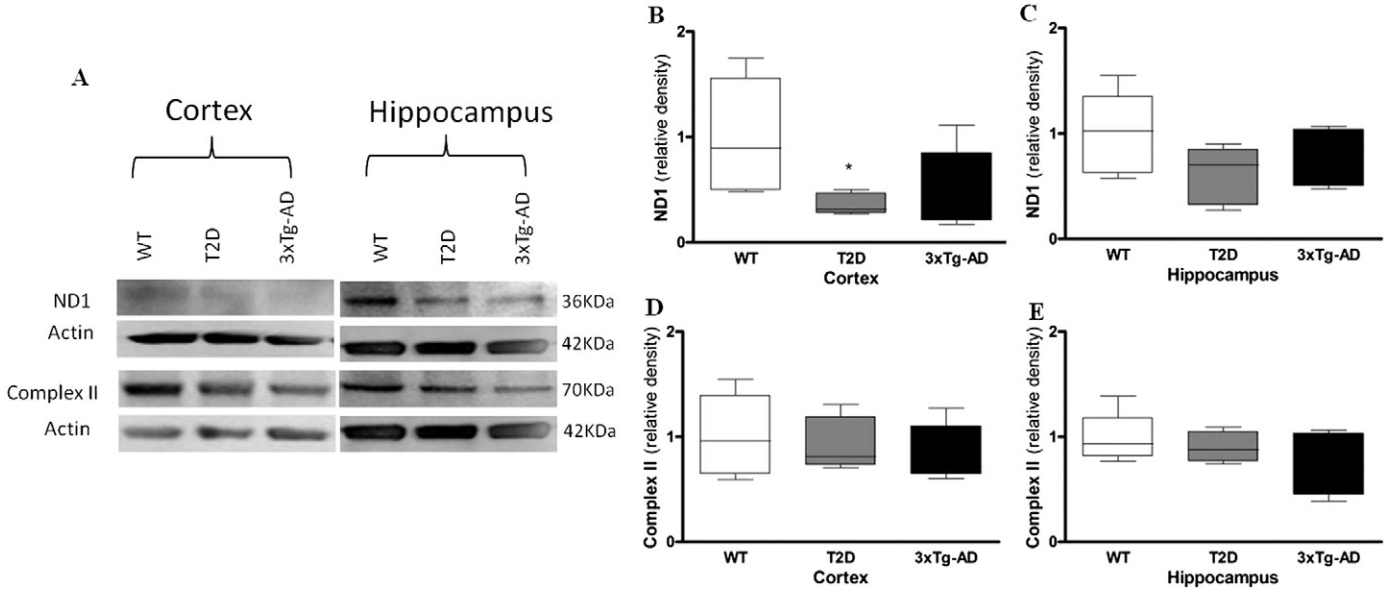

Fig. 6. T2D and AD effects in ND1 and 70KDa complex II subunits of mitochondrial respiratory chain complexes. Western blot representative images (A), cortex (B) and hippocampus (C) ND1 and cortex (D) and hippocampus (E) complex II protein levels. Data shown represent mean ± SEM from 5–7 animals.

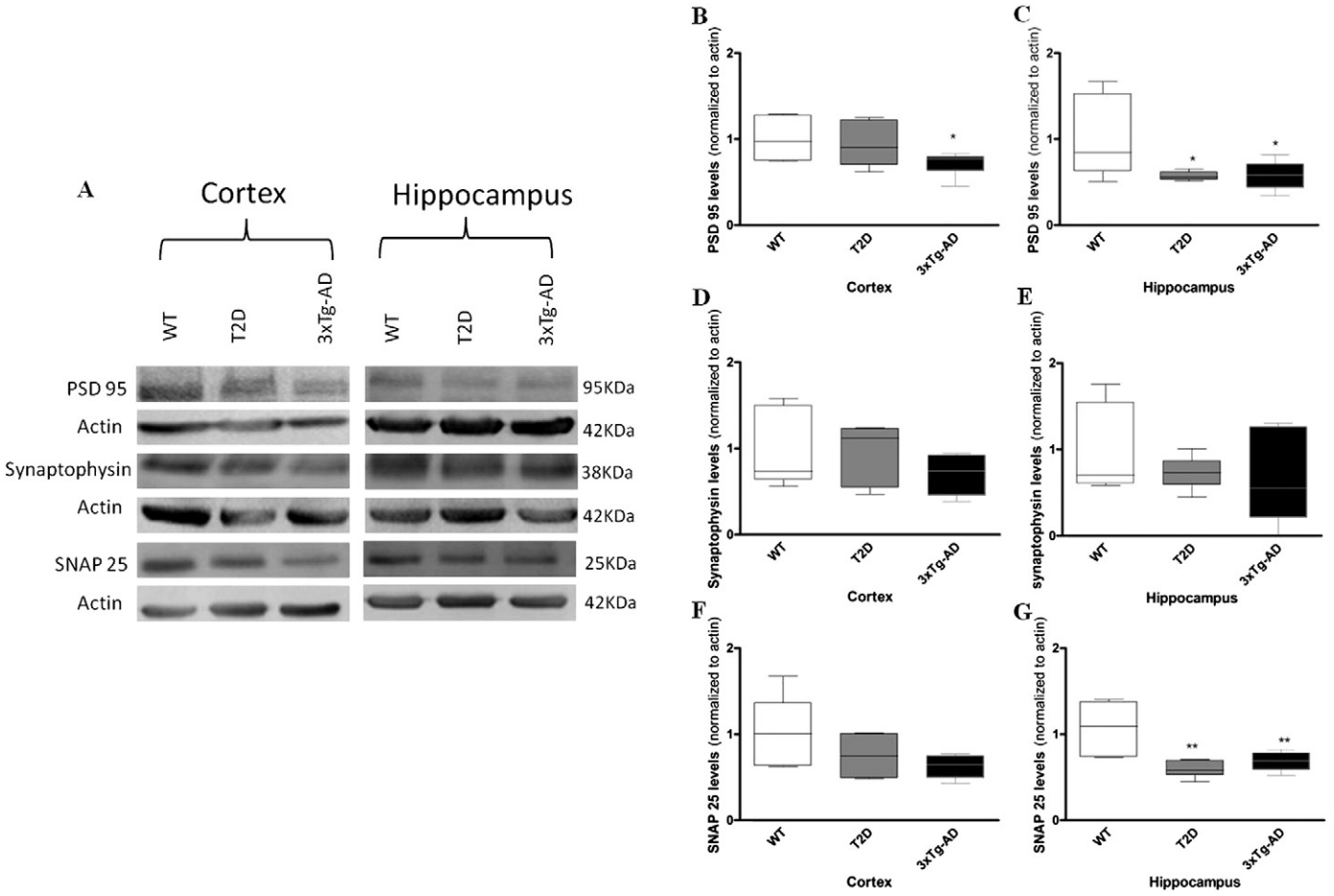

Fig. 7. T2D and AD effects in synaptic integrity. Western blot representative images (A), cortex (B) and hippocampus (C) PSD-95, cortex (D) and hippocampus (E) synaptophysin and cortex (D) and hippocampus (E) SNAP 25 protein levels. Data shown represent mean ± SEM from 5–7 animals.

decrease in the levels of synaptophysin was observed (Fig. 7D and E). Accordingly, Gylys and coworkers reported that the decrease in PSD95 levels (19%) was greater than that of synaptophysin in APPsw (Tg2576) transgenic mice and in AD patients' temporal cortex [75]. Synaptophysin, whose function remains unclear, seems to be necessary for kinetically efficient endocytosis of synaptic vesicles in neurons [77]. Similar to our observations (Fig. 7F and G), other studies demonstrate a decrease in SNAP25 levels in the brain of AD patients [78]. SNAP25 participates in docking and/or fusion of synaptic vesicles with the plasmalemma, a process essential for synaptic vesicle exocytosis [78]. It was previously reported that high levels of glucose decrease SNAP25 levels in hippocampal neurons while no alterations were observed in synaptophysin levels [79]. The alterations in synaptic integrity are associated with deficits in learning and memory, as previously shown [25].

Altogether our results show that the cerebral cortex and hippocampus of T2D and 3xTg-AD mice are characterized by mitochondrial function and biogenesis and autophagy impairments contributing to the loss of synaptic integrity. These observations reinforce the idea that T2D increases the risk of developing cognitive deficits and AD and suggest that mitochondrial biogenesis and autophagy may represent important targets for therapeutic intervention.

Transparency document

The Transparency document associated with this article can be found, in the version.

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