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Worsening of memory deficit induced by energy-dense diet in a rat model of early-Alzheimer's disease is associated to neurotoxic Aβ species and independent of neuroinflammation

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ABSTRACT

Diet is a modifiable risk factor for Alzheimer's disease (AD), but the mechanisms linking alterations in peripheral metabolism and cognition remain unclear. Since it is especially difficult to study long-term effects of high-energy diet in individuals at risk for AD, we addressed this question by using the McGill-R-Thy1-APP transgenic rat model (Tg(+/−)) that mimics presymptomatic AD. Wild-type and Tg(+/−) rats were exposed during 6 months to a standard diet or a Western diet (WD), high in saturated fat and sugar. Results from peripheral and hippocampal biochemical analysis and in situ respirometry showed that WD induced a metabolic syndrome and decreased presynaptic bioenergetic parameters without alterations in hippocampal insulin signaling or lipid composition. Cognitive tests, ELISA multiplex, Western blot, immunohistochemistry and RT-qPCR indicated that WD worsened cognition in Tg(+/−) rats, increased hippocampal levels of monomeric Aβ isoforms and oligomeric species, promoted deposits of N-Terminal pyroglutamate-Aβ (AβN3(pE)) in CA1 pyramidal neurons and interneurons, decreased transcript levels of genes involved in neuroprotective pathways such as Sirtuin-1 and increased nitrated proteins. Our results support the concept that in the presence of early Aβ pathology, diet-induced metabolic dysfunctions may contribute as a "second hit" to impair cognition. Noteworthy, such effect is not mediated by higher microglia activation or disruption of blood brain barrier. However, it may be attributed to increased amyloidogenic processing of amyloid precursor protein, generation of AβN3(pE) and dysregulation of pathways governed by Sirtuin-1. This evidence reinforces the implementation of prophylactic interventions in individuals at risk for AD.

1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia in older adults and represents a serious medical, social and economic problem. The 2015 World Alzheimer Report estimates that there are almost 900 million people aged over 60 living worldwide and predicts that between 2015 and 2050, the number of older people is expected to increase by 56% in high-income and by 239% in low income-countries. Studies aimed to evaluate the impact of modifiable risk factors are justified by their potential for prevention at a population level. The non-genetic risk factors with strongest evidence for possible association with dementia are poor early education, middle-aged hypertension, smoking

Abbreviations: 3-TyrNO$_2$, 3-nitrotyrosine; AβN3(pE), pyroglutamate-Aβ; iAβ, intraneuronal Aβ; BBB, blood-brain barrier; BHI, Bioenergetic Health Index; CRTC, CREB-regulated transcription coactivator; IDE, insulin degrading enzyme; IR, insulin resistance; MetS, metabolic syndrome; OCR, oxygen consumption rate; PGC-1α, PPARγ coactivator 1α; SD, standard diet; Sirt1, Sirtuin 1; SO, stratum oriens; SOD, superoxide dismutase; SP, stratum pyramidale; SRC, spare respiratory capacity; T2DM, type 2 diabetes mellitus; WAT, white adipose tissue; WD, Western diet.

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and type 2 diabetes (T2DM) across the lifespan. Hypertension and T2DM co-occur in middle-aged and older adults and are strongly influenced by dysregulation of insulin signaling, which starts with insulin resistance and is followed by hyperinsulinemia, metabolic syndrome (MetS) and finally T2DM [27]. MetS is defined as a meeting criterion for any three of the following: hyperglycemia, hypertension, hypertriglyceridemia, low high-density lipoprotein and/or abdominal obesity and it is highly dependent of hypercaloric diet [43].

Energy dense diets, high in saturated fat and sugar, are often referred to as Western diets (WD) based on their widespread popularity in Western and Westernized societies. Previous studies in humans and animal models have shown that consuming WD is not only associated with weight gain and metabolic syndrome, but also with impaired hippocampal-dependent memories and the emergence of hippocampal pathologies [14]. Hippocampus is one of the brain regions in AD to show amyloid β (Aβ) deposition and neurofibrillary tangles, possibly associated with cognitive impairment. The mechanisms underlying hippocampal amyloidosis, its contribution to progressive protein-aggregation pathology and neuronal loss observed at later stages of AD remain unclear. Recently, it has been demonstrated that cognitive decline and affective disorders are more pronounced in AD patients with MetS compared with those without, suggesting that insulin resistance (IR) and vascular endothelial dysfunction are strongly correlated with AD before brain pathological changes could be observed [26]. In this context, the hypothesis that MetS might operate as a "second hit" is suggestive as a potential trigger of AD progression. Prior research has established a clear relationship between obesity, IR, T2DM and dementia [78]. Neuroinflammation, Aβ generation and tau protein hyperphosphorylation, relocalization and deposition, considered as leading mechanisms in AD, are further propagated by obesity, MetS and T2DM [74]. In addition, it was recently shown that pyroglutamate-Aβ (AβN3(pE)), a major N-truncated/modified constituent of intracellular, extracellular, and vascular Aβ deposits present in AD and Down syndrome brain [62], is also present in the brain of cholesterol-enriched diet-fed rabbits [53]. This experimental evidence suggests that AβN3(pE) is a potential seeding unit that may be generated after exposure to an unhealthy diet and may play an important role in the formation of pathological amyloid aggregates. However, the complex and interacting mechanisms bridging metabolic dysfunction and cognitive impairment are not yet completely understood and require further investigation.

Experimental evidence indicates that Sirtuin 1 (Sirt1), an evolutionary conserved NAD^+-dependent deacetylase [9], is a key enzyme that controls metabolic status. Sirt1 constitutes a crucial molecular link between aging and human degenerative disorders. Consistent with this notion, Sirt1 is upregulated by various biological stressors, such as caloric restriction (which has been shown to prevent AD) and by neurotoxic conditions, event that may be interpreted as a neuroprotective adaptation response [33]. At the molecular level, Sirt1 targets different metabolic and anti-inflammatory pathways, among which transcription factors PGC-1α (PPARγ coactivator 1α) and CRTC (CREB-regulated transcription coactivator) are critical in neuronal metabolism and excitability, synaptic plasticity and in the acquisition of hippocampal-dependent memories [37,56,59]. However, no clear picture of how hippocampal Sirt1 expression influences brain energy metabolism and AD pathology has yet emerged. We and others have recently demonstrated that cognitive decline in AD is associated with decreased levels of genes regulated by Sirt1 such as CRTC, PGC-1α and Insulin Degrading Enzyme (IDE) [36,49].

Since it is especially difficult to study the mechanisms involved in long-term effects of high-energy diet in human subjects at risk for AD, we have chosen a transgenic rat model of early AD (McGill-R-Thy1-APP) to further investigate the underlying molecular events linking peripheral metabolism, brain pathology, presynaptic energy metabolism and cognitive dysfunction. The McGill-R-Thy1-APP hemizygous transgenic rat captures the full array of presymptomatic AD pathology, including intraneuronal Aβ (iAβ) accumulation, presynaptic bioenergetics deficit and working and reference memory impairments from adulthood (6 months) to middle age (12 months).

In the present study, we have hypothesized that long-term WD promotes the generation of neurotoxic Aβ in the hippocampus of Tg(+/−) rats and downregulates cellular programs believed to provide cell protection against neurotoxic insults , thereby contributing as a "second hit" to aggravate cognitive impairment.

2. Material and methods

2.1. Animals and nutritional protocols

Hemizygous transgenic McGill-R-Thy1-APP (Tg(+/−)) rats harboring the human APP751 transgene with the Swedish and Indiana mutations under the control of the murine Thy1.2 promoter were generated using HsdBrI:WH Wistar strain [38] Animals were provided to FIL by The Royal Institution for the Advancement of Learning/McGill University, Montreal, Quebec (Canada). An in-house colony was established at FIL as previously described [19]. Nine Tg(+/−) rats and 10 wild-type (WT) controls were individually maintained in polycarbonate cages in a temperature-controlled animal facility with a 12-h dark/light cycle and allowed to consume standard diet (SD) and water ad libitum, while 8 Tg(+/−) and 13 WT rats consumed a Western diet (WD), a diet high in saturated fat and fructose, from 35 days old to euthanization (approximately six and a half months old). A detailed composition of SD and WD, caloric density and percentage of energy are described in Table 1. Adequate levels of essential nutrients were provided.

2.2. Total blood glucose, insulin, cholesterol and triglycerides measurements

Every 6 weeks rats were fasted overnight and baseline blood samples (150–200 μL) were collected from the tail vein into microcentrifuge tubes. Blood glucose, insulin and fasting triglyceride and cholesterol levels were assayed as described in Supplementary File 1.

Table 1
Composition of standard chow and hypercaloric diet.

	Diet	
	Standard (SD)	Western (WD)
Nutrient (%)		
Carbohydrate	72.25	56.25
Dextrinized cornstarch		
Protein		
Casein	14	14
Fat		
Sunflower oil	4	
Beef fat		20
Fiber		
Cellulose	5	5
Mineral mix		
AIN 93M-MX	3.5	3.5
Vitamin mix		
AIN 93-VX	1	1
Choline bitartrate	0.25	0.25
Water ad libitum	+	+
Fructose supplementation	–	6.5–8 g/day
Energy (kcal/100 g)	381	461
From carbohydrates (%)	76	49
From protein (%)	15	12
From fat (%)	9	39

2.3. Cognitive assessment

When animals reached 6 months of age, neurological reflexes, spatial learning, working and reference memory were assessed. We only performed tests that had previously shown differences between Tg(+/−) and WT animals [19]. Description of each test can be found in Supplementary File 1.

2.4. Analysis of body adiposity

Weight gain was calculated by subtracting the final weight from the initial weight. After euthanasia, adiposity measures were obtained from retroperitoneal, epididymal and visceral fat masses. Retroperitoneal white adipose tissue (WAT) was expressed as a percentage of body weight.

2.5. Lipid composition in hippocampal samples

After completing cognitive evaluation, hippocampi were dissected, frozen with liquid nitrogen and preserved at −80 °C until used, as described [41]. Total lipids, total triglycerides, total phospholipids and total cholesterol were determined as detailed in Supplementary File 1.

2.6. Hippocampal expression of Aβ isoforms

To quantify soluble Aβ 38/40/42, MSD® V-PLEX PLUS Aβ Peptide Panel 1 kit was used as detailed in Supplementary File 1.

2.7. Hippocampal levels of Aβ oligomers, APP-CTFβ fragments, insulin signaling, synaptic and nitrated proteins

Isolated hippocampi were homogenized and protein extracts were extracted using illustra TriplePrep Kit (GE Healthcare) and analyzed by Western blot as described in Supplementary File 1.

2.8. Presynaptic bioenergetic studies

Synaptosomes were isolated, characterized and cryopreserved as reported [41]. Oxygen consumption assays were performed as detailed in Supplementary File 1. Bioenergetic Health Index (BHI) was calculated using the following formula: BHI = spare respiratory capacity × ATP-dependent respiration / non-mitochondrial respiration × proton leak [13].

2.9. Evaluation of hippocampal Aβ pathology and brain inflammation

Brain sections were analyzed by using systematic random sampling. Every sixth brain section (240 μm apart) was stained using a free-floating immunohistochemistry procedure [38]. Intraneuronal Aβ (iAβ), N-terminal truncated/modified Aβ species (AβN3(pE)), activated microglia and brain microvessels were detected as described in Supplementary File 1.

2.10. Evaluation of the expression of energy metabolism related genes in hippocampi of 6 month-old rats

RNA from hippocampal homogenates was isolated using illustra TriplePrep Kit (GE Healthcare) and transcript levels of *SIRT1*, *PGC1α* and *superoxide dismutase 2* (*SOD2*) genes measured as detailed in Supplementary File 1.

2.11. Statistical analysis

All data are shown as the mean ± SEM of at least three independent experiments except for behavioral assessments where experiments were carried out once. Data were analyzed by unpaired two-tailed Student's *t*-test, one or two-way ANOVA tests or by three-way mixed ANOVA tests followed by Tukey's HSD post-hoc tests for multiple comparisons. When normality and/or homoscedasticity assumptions were not met, Kruskal-Wallis tests followed by post-hoc Mann-Whitney tests adjusted by Bonferroni correction were employed. The significance level was set at $p \leq 0.05$. SPSS 15.0 for Windows software (Chicago, IL, USA) was used to perform all statistical analyses.

3. Results

3.1. Western diet (WD) impacts on peripheral metabolic parameters in both WT and Tg(+/−) rats

In order to determine the effects of a WD on metabolic parameters in a rat model of early AD, male WT and Tg(+/−) rats were fed with either SD or WD. Treatment started at 35 days of age and finished after behavioral evaluation at 6 months of age. This specific time point was chosen because we have previously reported that 6 month-old Tg(+/−) rats show neuropathology, cognitive impairment and synaptic bioenergetic deficiencies similar to early AD [19,41]. We did not find diet-induced obesity in WD-treated groups (Fig. 1 A). However, significant differences were detected in retroperitoneal white adipose tissue (WAT), systolic blood pressure, fasting serum triglycerides levels, fasting serum cholesterol levels and fasting total blood glucose between SD- and WD-treated groups (Fig. 1 B–F). As previously reported in a transgenic mouse model of AD [61], Tg(+/−)-SD rats showed significantly higher systemic insulin levels than WT-SD rats, and WD exacerbates this phenotype with no impact in WT animals (Fig. 1 G). An additional sign pointing to the possible presence of MetS in 6 month-old Tg(+/−)-WD rats was a significant decrement in the activation state of the signaling kinase Akt in liver homogenates (Fig. 1 H) in agreement with peripheral IR. Conversely, there were no changes neither in the hippocampal ratio of phosphorylated Akt/total Akt (Fig. 1 I, left panel) nor in the expression of insulin receptor (Fig. 1 I, right panel) in response to liver IR and/or WD.

3.2. Western diet (WD) worsens cognitive performance of Tg +/− rats

To assess whether WD worsens cognitive performance of Tg(+/−) rats, we employed the spontaneous alternation task (Y-maze) and the Morris water maze (MWM). In the Y-maze, all groups showed a similar exploration activity operationalized as the total number of arm entries (Fig. 2 A, left). However, analysis of the spontaneous alternation behavior indicated that WT groups did not differ according to diet, while the Tg(+/−)-SD group displayed a significantly lower percentage of alternations than WT groups (Fig. 2 A, right). Moreover, Tg(+/−)-WD group showed the lowest percentage of alternations (Fig. 2 A, right), suggesting that WD worsens the spatial working memory deficit observed in Tg(+/−)-SD rats. In the MWM, all groups reduced their escape latencies and paths length to a similar level during the second day of the cued learning, indicating that none of the groups was visually impaired or showed deficits in swimming abilities (Fig. 2 B–C). During the spatial learning training, Tg(+/−)-WD group exhibited significantly higher escape latencies and longer paths length than the other groups during days 3 and 4, while tendencies to significance were observed during the last day (Fig. 2 B–C). All groups showed similar swimming speeds during cued and spatial learning training (Supplementary File 2), suggesting similar levels of motivation. In the probe trial, WT groups spent a significantly higher percentage of time in the target quadrant than expected by chance, regardless of the type of diet, while Tg(+/−) groups did not (Fig. 2 D). Furthermore, Tg(+/−) groups crossed the platform zone significantly fewer times and spent a significantly less percentage of time in the circular predefined zone than WT groups (Fig. 2 E). These results show that WD does not impair cognition in

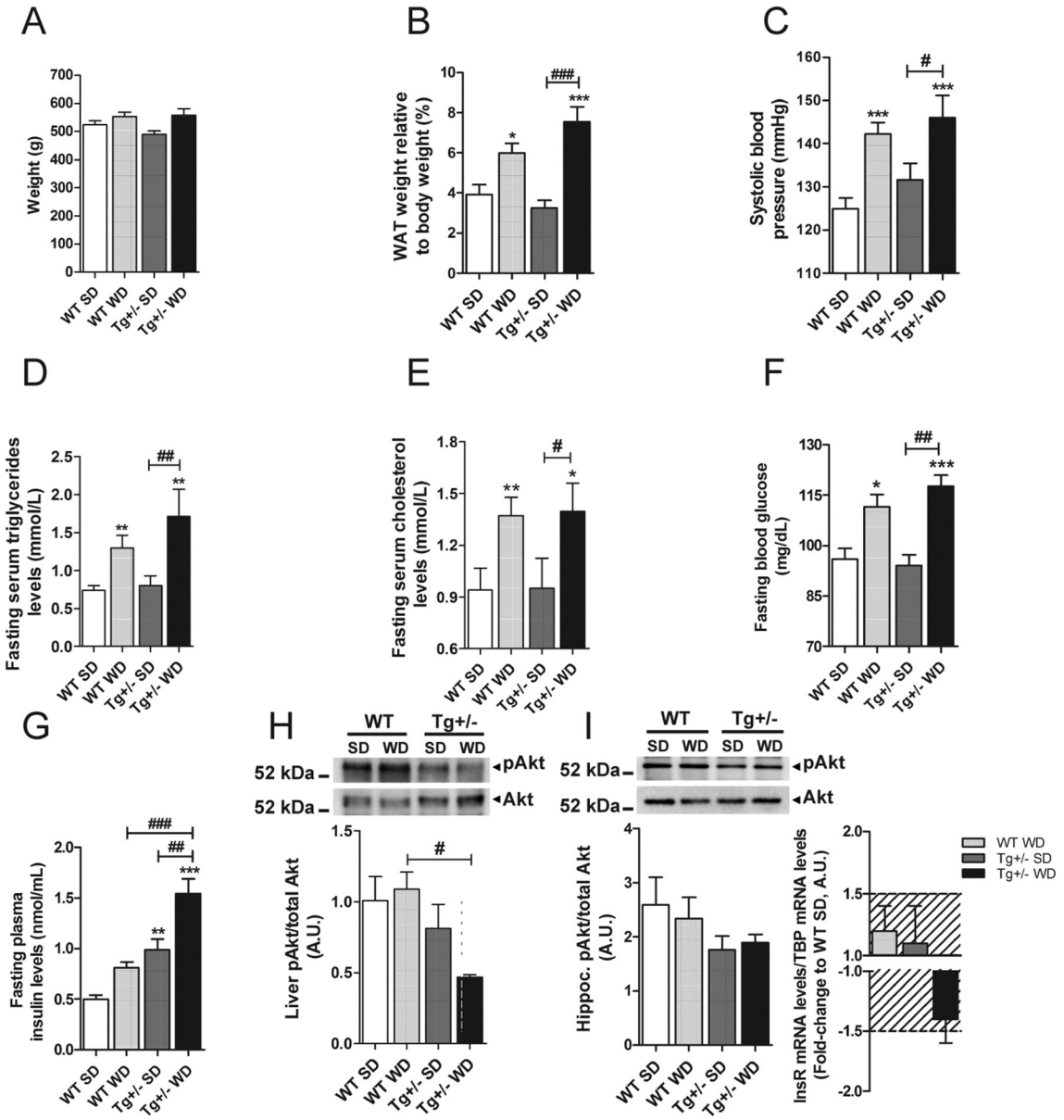

Fig. 1. Long-term Western diet induces metabolic syndrome in male rats. Bars show (A), body weight (Genotype: $F_{(1, 36)} < 1$; Diet: $F_{(1, 36)} < 1$, Interaction: $F_{(1, 36)} < 1$). (B), White adipose tissue (WAT) weight relative to body weight (Genotype: $F_{(1, 36)} < 1$; Diet: $F_{(1, 36)} = 29.25$, $p < 0.001$; Interaction: $F_{(1, 36)} = 3.55$, $p =$ n.s.). (C), Systolic blood pressure (Genotype: $F_{(1, 36)} = 1.92$, $p =$ n.s.; Diet: $F_{(1, 36)} = 17.94$, $p < 0.001$; Interaction: $F_{(1, 36)} < 1$). (D), Fasting serum triglycerides (Genotype: $F_{(1, 36)} = 1.30$, $p =$ n.s.; Diet: $F_{(1, 36)} = 12.53$, $p < 0.01$; Interaction: $F_{(1, 36)} < 1$). (E), Fasting serum cholesterol levels (Genotype: $F_{(1, 36)} < 1$; Diet: $F_{(1, 36)} = 9.32$, $p < 0.01$; Interaction: $F_{(1, 36)} < 1$). (F), Fasting blood glucose levels (Genotype: $F_{(1, 36)} < 1$; Diet: $F_{(1, 36)} = 40.27$, $p < 0.001$; Interaction: $F_{(1, 36)} < 1$) and (G), fasting plasma insulin levels (Genotype: $F_{(1, 36)} = 48.54$, $p < 0.001$; Diet: $F_{(1, 36)} = 24.76$, $p < 0.001$; Interaction: $F_{(1, 36)} < 1$) at the end of treatment (6 months old) in WT and Tg(+/−) rats fed with SD or WD. Liver (H) (Genotype: $F_{(1, 8)} = 9.29$, $p = 0.01$; Diet: $F_{(1, 8)} < 1$; Interaction: $F_{(1, 8)} = 2.47$, $p =$ n.s.), and hippocampal (I, left panel) (Genotype: $F_{(1, 8)} = 3.30$, $p =$ n.s.; Diet: $F_{(1, 8)} < 1$; Interaction: $F_{(1, 8)} < 1$) pAkt/Akt ratio in animals from (G). (I) right panel, insulin receptor (InsR) transcript levels in animals from (G). Each bar represents the mean value of at least three independent experiments performed by triplicate for each sample normalized by TATA-binding protein (TBP). The mean ± SEM relative to WT-SD (=1) is shown. Values between dashed lines (+1.5 and −1.5) were considered not different from WT-SD (=1). Data are expressed as mean ± SEM. Post-hoc tests: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. WT SD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. WT-SD and WT-WD, wild-type rats fed with either standard (SD) or Western diet (WD); Tg(+/−)-SD and Tg(+/−)-WD, transgenic rats fed with either SD or WD.

WT while worsens cognitive dysfunction in Tg(+/−) animals (detailed statistical analysis in Supplementary File 3).

3.3. Western diet (WD) does not affect lipid composition, neuroinflammation or synaptic structure in the hippocampus

To test whether the deterioration of hippocampal-dependent memories was associated with hippocampal structural and/or functional alterations we determined lipid composition, synaptic integrity, blood-brain barrier (BBB) permeability, and neuroinflammation. Our results showed no significant differences among groups in the amount of lipids (Supplementary File 4), expression levels of presynaptic/postsynaptic proteins (Supplementary File 5), extravasated plasma-derived IgG, TNF-α and IL-1β levels (Fig. 3 A–C). In addition, similar number of capillary endothelial cells and absence of activated microglia were observed in Tg(+/−)-SD and WD rats (Fig. 3 D).

3.4. Negative impact of Western diet (WD) on synaptosomal bioenergetics of Tg(+/−) and WT rats

Since we have previously reported that Tg(+/−) rats under conditions of energetic load show presynaptic mitochondrial dysfunction

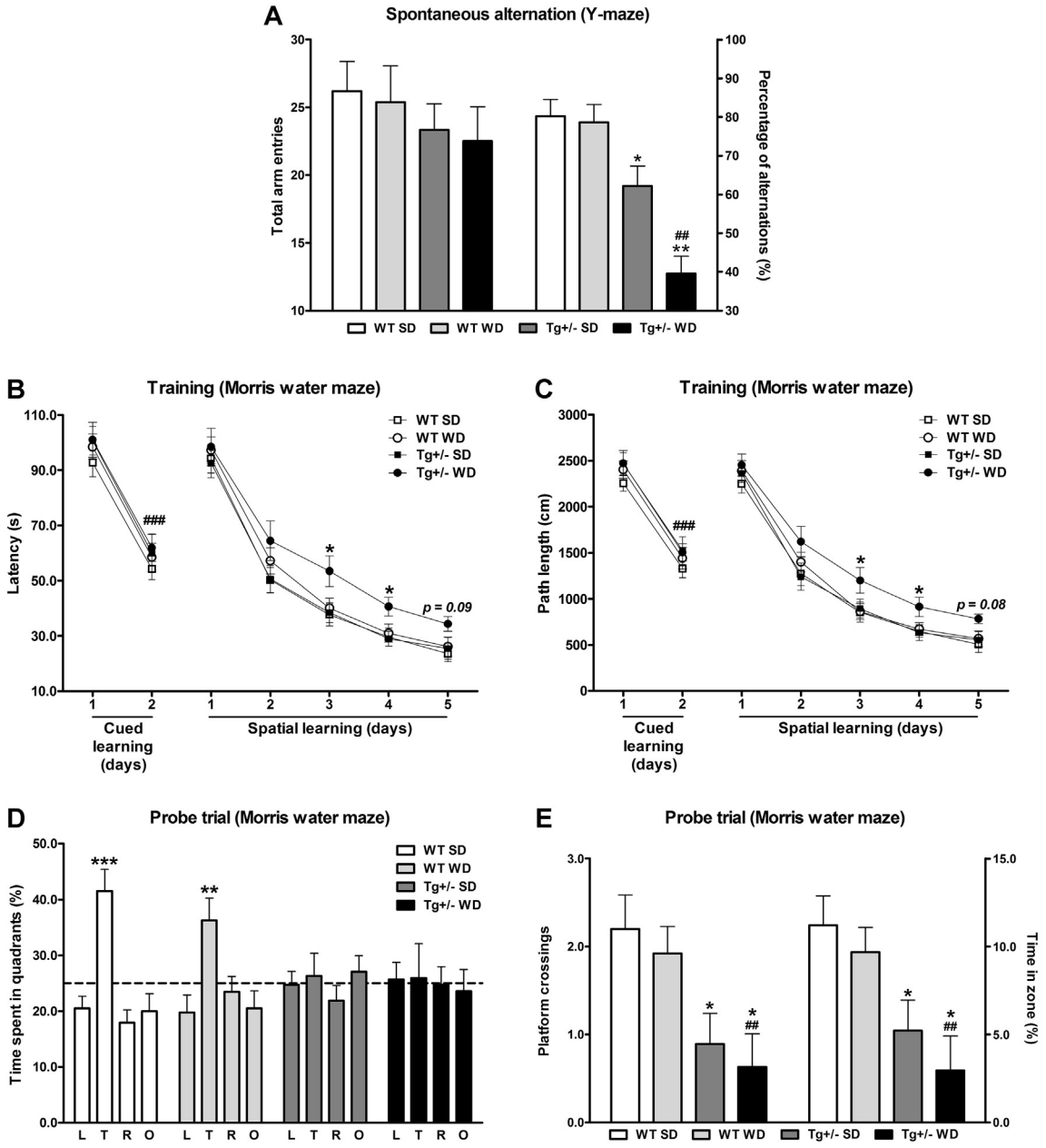

Fig. 2. Long-term Western diet worsens the cognitive deficits exhibited by Tg(+/−) rats. (A), All of the groups showed similar levels of exploration but WD worsened the spatial working memory deficit showed by Tg(+/−) rats. Post-hoc tests: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. WT groups; $^{\#\#}p < 0.01$ vs. Tg(+/−)-SD group. (B-C), All of the groups displayed a similar performance in the cued learning task, while WD delays spatial learning in Tg(+/−) rats. Post-hoc tests: $^{\#\#\#}p < 0.001$ vs. day 1; $^{*}p < 0.05$ vs. all other groups. (D), Tg(+/−) groups spent a similar percentage of time in the target quadrant than expected by chance (dashed line, 25%), indicating that Tg(+/−) rats have an impairment in spatial reference memory regardless of the type of diet (one-sample t-tests: WT-SD: $t = 4.27$, $df = 9$, $^{***}p < 0.001$; WT-WD: $t = 2.78$, $df = 12$, $^{**}p = 0.01$; Tg(+/−)-SD: $t = 0.32$, $df = 8$, p = n.s.; Tg(+/−)-WD: $t = 0.16$, $df = 7$, p = n.s.). L, left quadrant; T, target quadrant, R, right quadrant, O, opposite quadrant. (E), Regardless of the type of diet, Tg(+/−) groups crossed fewer times the platform zone and spent less percentage of time in the predefined circular zone larger than platform, reinforcing the idea that Tg(+/−) groups showed an impairment in spatial reference memory. Post-hoc test: $^{*}p < 0.05$ vs. WT groups; $^{\#\#}p < 0.01$ vs. WT-SD group. Data are expressed as the mean ± SEM. WT-SD and WT-WD, wild-type rats fed with either standard (SD) or Western diet (WD); Tg(+/−)-SD and Tg(+/−)-WD, transgenic rats fed with either SD or WD.

[41], we evaluated whether WD worsens this phenotype. At the end of the nutritional protocol we determined the oxygen consumption rate (OCR) profiles of synaptosomes. When compared to WT-SD rats, all experimental groups showed diminished spare respiratory capacity (SRC) and altered non-mitochondrial respiration, while no significant changes in ATP-dependent respiration and proton leak were detected (Table 2). However, after the integration of these parameters to calculate the BHI, both WT-WD and Tg(+/−)-WD evidenced lower BHI values compared to WT-SD (Table 2), suggesting that WD impacts negatively on presynaptic global bioenergetics regardless of the genotype.

3.5. Western diet (WD) alters pathways related to neuroprotection and cell energy regulation promoting nitroxidative stress

To determine whether bioenergetic failure was associated with downregulation of genes involved in brain metabolism and detoxification of oxidative species, the transcript levels of SIRT1, PGC-1α and SOD2 were determined by RT-qPCR. Our results showed that in 6 month-old Tg(+/−)-SD, levels of Sirt1 mRNA increased 25-fold compared to WT-SD (Fig. 4 A), suggesting that in Tg(+/−) animals, neurotoxic damage mediated by iAβ triggers Sirt1 expression. However, after

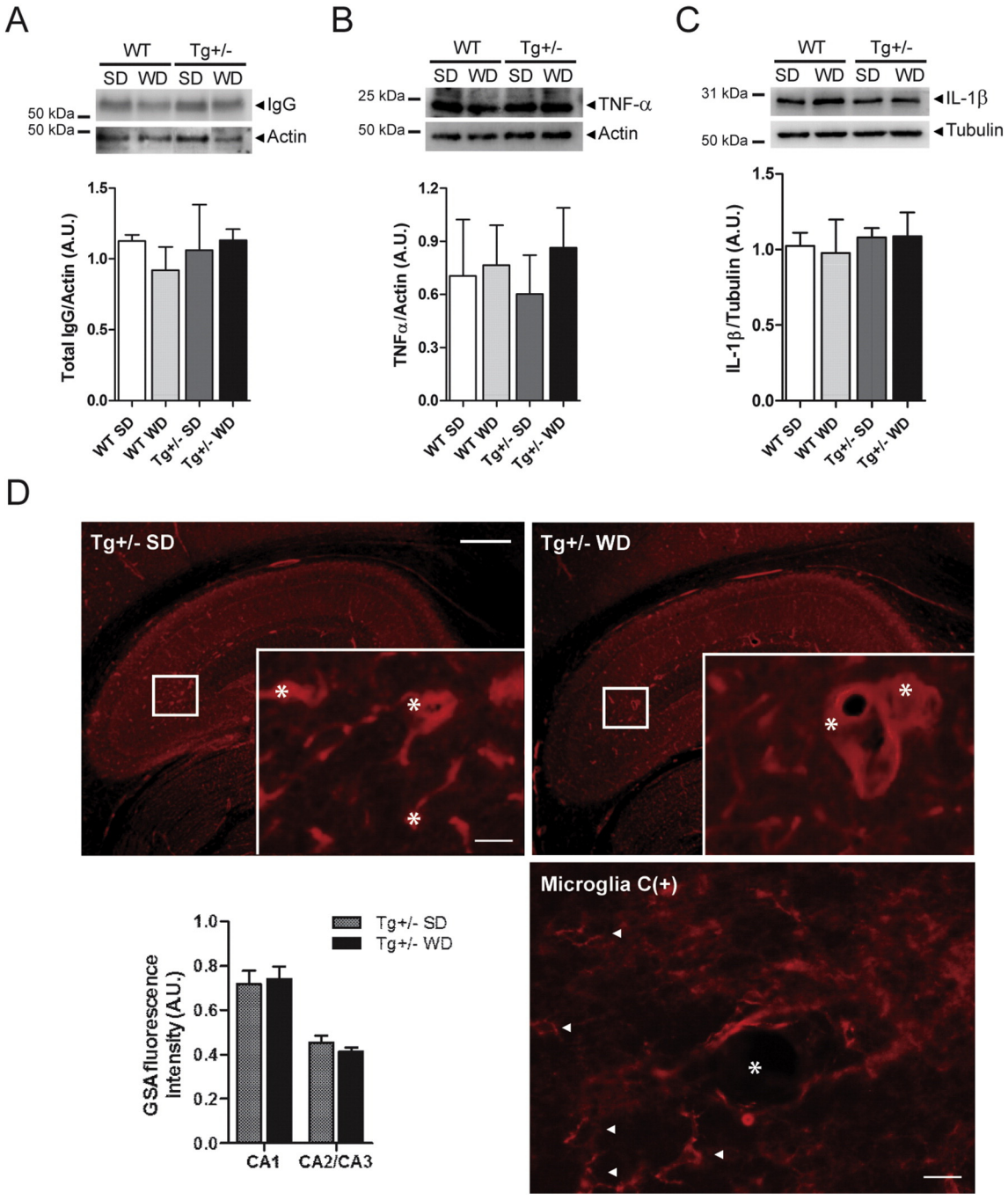

Fig. 3. Western diet does not generate blood-brain barrier (BBB) disruption or neuroinflammation in the hippocampus. (A), (B), and (C), upper panels, representative immunoblots of extravasated plasma-derived IgG, TNF-α and IL-1β in the hippocampus of WT and Tg(+/−) rats fed with SD or WD normalized with actin or tubulin levels. Left, molecular weight markers. Lower panels, bars show the mean ± SEM of each protein level normalized by actin or tubulin expressed as arbitrary units (A.U.). No significant differences were detected among groups (IgG, Genotype: $F_{(1,\ 8)} < 1$; Diet: $F_{(1,\ 8)} < 1$; Interaction: $F_{(1,\ 8)} < 1$; TNF-α, Genotype: $F_{(1,\ 8)} < 1$; Diet: $F_{(1,\ 8)} < 1$; Interaction: $F_{(1,\ 8)} < 1$; IL-1β, Genotype: $F_{(1,\ 8)} < 1$; Diet: $F_{(1,\ 8)} < 1$; Interaction: $F_{(1,\ 8)} < 1$). (D), Upper panels, representative photomicrographs in the hippocampus of 6-month-old rats fed with SD (left) or WD (right) showing immunofluorescence images of endothelial microvessels (asterisk) stained with biotinylated-*Griffonia simplicifolia* agglutinin-I (GSA-I) and developed with Cy3-streptoavidin. Activated microglia was not detected in Tg(+/−) under SD or WD. Lower left panel, bars show the mean ± SEM level of GSA fluorescence intensity of endothelial microvessels in CA1 and CA2/CA3 regions as expressed as arbitrary units (A.U.) (CA1: $t = 0.24$, p = n.s.; CA2/CA3: $t = 0.99$, p = n.s.). Lower right panel, positive control showing microglia activation (arrowheads) as a consequence of intracerebral injection of IL-1β. Scale bars, 500 μm (upper panels) and 50 μm (insets and lower right panel). WT-SD and WT-WD, wild-type rats fed with either standard (SD) or Western diet (WD); Tg(+/−)-SD and Tg(+/−)-WD, transgenic rats fed with either SD or WD.

long-term exposure to WD, Tg(+/−) animals showed similar levels of Sirt1 mRNA compared to WT-SD (Fig. 4 A), suggesting that WD negatively impacts on Sirt1 expression and reduces the neuroprotective program triggered by Sirt1. A similar profile was obtained for SOD2 expression, which was increased almost 10-fold in Tg(+/−)-SD compared to WT-SD and after WD exposure it was reduced 5-fold (Fig. 4 A). Under basal conditions, PGC-1α showed a 1.8-fold reduction compared to WT-SD and an exacerbation of this decrement (>10-fold) was detected after exposure to WD (Fig. 4 A). It is of note that reduced Sirt1 mRNA levels did not impact on CRTC1 hippocampal protein content (Supplementary File 6), suggesting that protein levels of this downstream gene of Sirt1, associated with learning deficits in

Table 2
Bioenergetic parameters of isolated synaptosomes from 6 month-old animals.

	WT rats		Tg(+/−) rats	
	SD %	WD % (IQR)	SD % (IQR)	WD % (IQR)
SRC	100	61.7 (56.6–89.1)*	64.3 (54.9–81.3)*	47.2 (11.7–55.1)*
ATP-depend. resp.	100	94.0 (90.2–100.6)	96.0 (88.6–114.8)	61.8 (57.6–137.4)
Proton leak	100	89.4 (64.5–111.4)	99.8 (57.4–100.0)	69.5 (60.7–119.5)
Non-mito resp.	100	133.8 (112.6–160.9)*	65.7 (64.9–91.3)*	105.1 (102.7–111.1)*
BHI	100	61.4 (38.3–75.8)*	95.2 (87.6–167.1)	41.2 (11.6–51.6)*

SRC, spare respiratory capacity; ATP-depend. resp., ATP-dependent respiration; non-mito resp., non-mitochondrial respiration; BHI, Bioenergetic Health Index; SD, standard diet; WD, Western diet; IQR, interquartile range. Data are expressed as median and interquartile range relative to WT-SD (100%). Kruskal-Wallis tests, SRC: $H = 14.34$, $df = 3$, $p < 0.001$; ATP-depend. resp.: $H = 2.18$, $df = 3$, p = n.s.; proton leak: $H = 2.33$, $df = 3$, p = n.s.; non-mito resp.: $H = 15.35$, $df = 3$, $p < 0.001$; BHI: $H = 13.16$, $df = 3$, $p < 0.001$.
* Mann-Whitney post-hoc tests (Bonferroni correction): $p < 0.05$ compared to WT-SD.

Tg(+/−) rats [40], remained unchanged with diet. Since SOD2 gene codes for Mn^{2+}-SOD, which is the enzyme responsible for detoxifying superoxide anion ($O_2^{\bullet -}$) in mitochondria, we wondered whether SOD downregulation could promote hippocampal protein damage mediated by an excess of $O_2^{\bullet -}$ derived oxidant species. In order to evaluate this hypothesis, we measured the levels of 3-nitrotyrosine in hippocampal

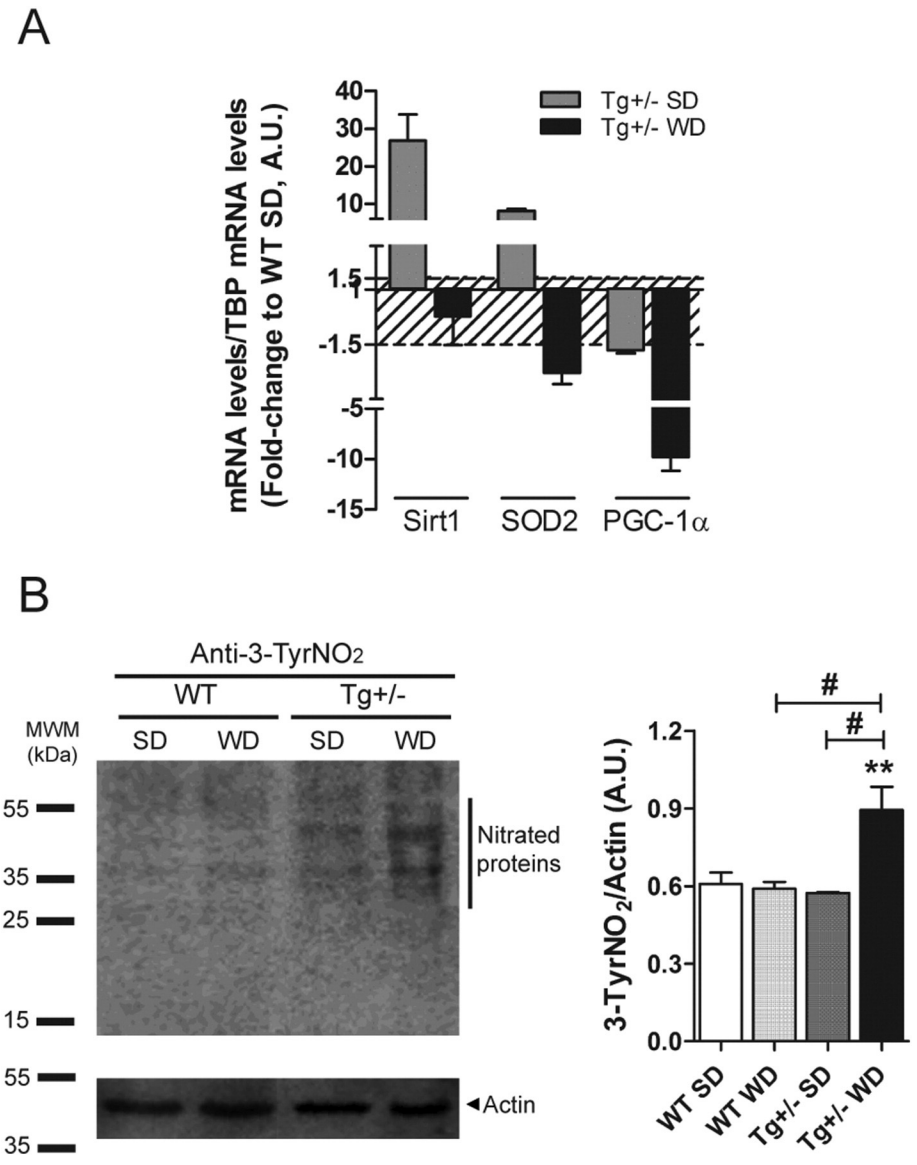

Fig. 4. Long-term Western diet alters neuroprotective pathways and promotes nitroxidative stress. (A), The endogenous Sirt-1, SOD2, and PGC-1α transcript levels were determined by RT-qPCR from hippocampal homogenates of WT-SD, Tg(+/−)-SD and Tg(+/−)-WD. Each bar represents the mean value of at least three independent experiments performed by triplicate for each sample normalized by TATA-binding protein (TBP). The mean ± SEM in Tg(+/−) relative to WT-SD (=1) is shown. Values between dashed lines (+1.5 and −1.5) were considered not different from WT-SD (=1). (B), Left panel, representative Western blot of hippocampal homogenates of WT and Tg(+/−) rats fed with either SD or WD probed with anti-3-nitrotyrosine (3-$TyrNO_2$) (upper panel) and actin (lower panel). Left, molecular weight markers. Right panel, bars show the levels of 3-$TyrNO_2$ proteins relative to actin content (Genotype: $F_{(1, 8)} = 6.66$, $p < 0.05$; Diet: $F_{(1, 8)} = 8.52$, $p < 0.05$; Interaction: $F_{(1, 8)} = 10.72$, $p = 0.01$). Data are expressed as mean ± SEM. Post-hoc tests: **$p < 0.01$ vs. WT-SD; #$p < 0.05$. WT-SD and WT-WD, wild-type rats fed with either standard (SD) or Western diet (WD); Tg(+/−)-SD and Tg(+/−)-WD, transgenic rats fed with either SD or WD.

homogenates from SD and WD-fed rats and found increments of nitrated proteins in Tg(+/−)-WD rats (Fig. 4B).

3.6. Western diet (WD) dramatically increases Aβ levels and generates pyroglutamate-Aβ deposits

To determine whether WD influences AD pathogenesis in this transgenic rat model, we evaluated amyloid precursor protein (APP) processing and Aβ levels in the hippocampus. Tg(+/−)-WD rats showed higher accumulation of a 12 kDa anti-Aβ/APP immunoreactive band, comprising Aβ trimers and APP-CTF fragments (Fig. 5 A). In addition, we performed a highly sensitive multiplex ELISA to quantify total Aβ levels within the hippocampus. Surprisingly, and despite the use of a mild amyloidosis model, we found that Aβ40 and Aβ42 were elevated nearly 3-fold in Tg(+/−)-WD vs. Tg(+/−)-SD hippocampi (Fig. 5 B). Moreover, Aβ38, which was below the range of detection in Tg(+/−)-SD brains, was accumulated in Tg(+/−)-WD hippocampus with a concentration of 8.68 ± 1.35 pg/mg of total protein. To address whether changes in Aβ levels were due to hippocampal upregulation of BACE1 gene expression and/or down regulation of IDE protein levels, we quantified BACE1 transcript levels by RT-qPCR and IDE protein levels by Western blot. No significant differences were observed among groups analyzed (Fig. 5 D and Supplementary File 7). Moreover, histochemical analysis of hippocampal iAβ immunoreactivity (Fig. 6 A) did not show differences between Tg(+/−)-SD and Tg(+/−)-WD animals. Based on previous observations suggesting that high-fat diet induces deposition of AβN3(pE), we probed hippocampal sections of Tg(+/−) rats with a specific anti-AβN3(pE) antibody. Interestingly, we found AβN3(pE)-positive neurons in CA1 region (2 ± 1/1.8 mm^2 of CA1) from Tg(+/−) WD rats, while no immunoreactivity was detected in Tg(+/−)-SD rats (Fig. 6 B). Neurons labelled with anti-AβN3(pE) were localized in either *stratum pyramidale* (SP) or *stratum oriens* (SO) of CA1 field, and consequently identified as pyramidal neurons or interneurons, respectively (Fig. 6 B).

4. Discussion

In this study, we have examined the impact of a long-term, high-energy diet on cognitive performance in rats and its relation to presynaptic bioenergetics, neuropathological changes and APP processing. We employed Tg(+/−) rats that show cognitive impairment similar to early AD as well as WT animals. While body adiposity, blood pressure, triglycerides, cholesterol and glucose levels were significantly elevated in WD-treated groups, IR at peripheral level was only found in Tg(+/−)-WD. It is of note that Tg(+/−)-SD rats displayed hyperinsulinemia in accordance with a previous report in male APP/PS1 transgenic mice [61]. It was proposed that central amyloidosis disrupts insulin signaling in the hypothalamus dysregulating peripheral glucose homeostasis, being hyperinsulinemia a compensatory mechanism to maintain glucose homeostasis. While the exact mechanisms underlying metabolic dysregulation in the CNS of Tg(+/−) rats remains to be elucidated, we demonstrated that WD exacerbates this phenotype. Noteworthy, we did not find evidence of hippocampal IR in WD-treated animals, suggesting that central IR may be independent of the amount of carbohydrates and saturated fat or the source in the diet. Our negative results are supported by ex vivo experiments, showing that high-energy

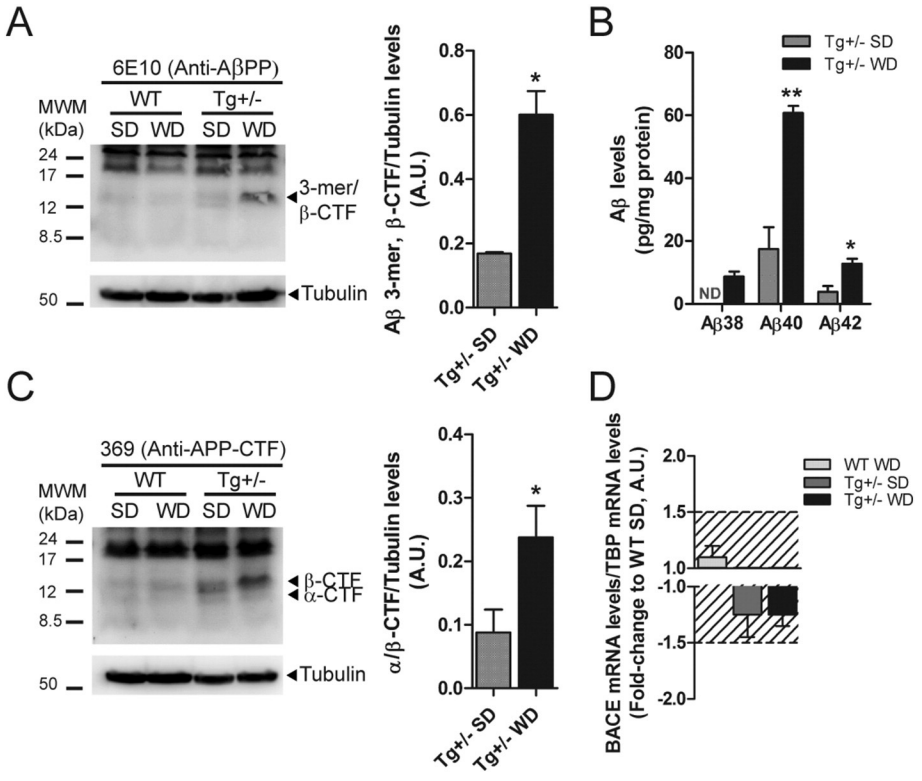

Fig. 5. Impact of Western diet on hippocampal APP processing and Aβ metabolism. Equal amount of hippocampal samples from 6 month-old Tg(+/−) and WT rats fed with SD or WD were resolved by SDS-PAGE and visualized by Western blotting with (A) a monoclonal antibody (6E10) that recognizes the human N-terminal sequence of Aβ and (C) a polyclonal antibody that detects APP-α/β-CTF (left panels). Tubulin levels indicate protein content. MWM, molecular weight markers. Arrows, in (A) Aβ trimers/APP-CTF fragments and in (B) APP-α/β-CTF. Bars in (A) and (C) show the mean ± SEM of immunoreactive bands expressed as arbitrary units (A.U.) ($t = 5.87$, $p < 0.05$; $t = 2.38$, $p < 0.05$, respectively). (B), Hippocampal Aβ38/40/42 levels quantified by high sensitive multiplex ELISA in SD and WD-fed rats ($t = 5.97$ $^{**}p < 0.005$, $t = 3.74$, $^{*}p < 0.05$). (D) BACE1 transcript levels from hippocampal homogenates of all groups analyzed. Each bar represents the mean value of at least three independent experiments performed by triplicate for each sample normalized by TATA-binding protein (TBP). The mean ± SEM relative to WT-SD (=1) is shown. Values between dashed lines (+1.5 and −1.5) were considered not different from WT-SD (=1). WT-SD and WT-WD, wild-type rats fed with either standard (SD) or Western diet (WD); Tg(+/−)-SD and Tg(+/−)-WD, transgenic rats fed with either SD or WD. $^{*}p < 0.05$ vs. Tg(+/−)-SD.

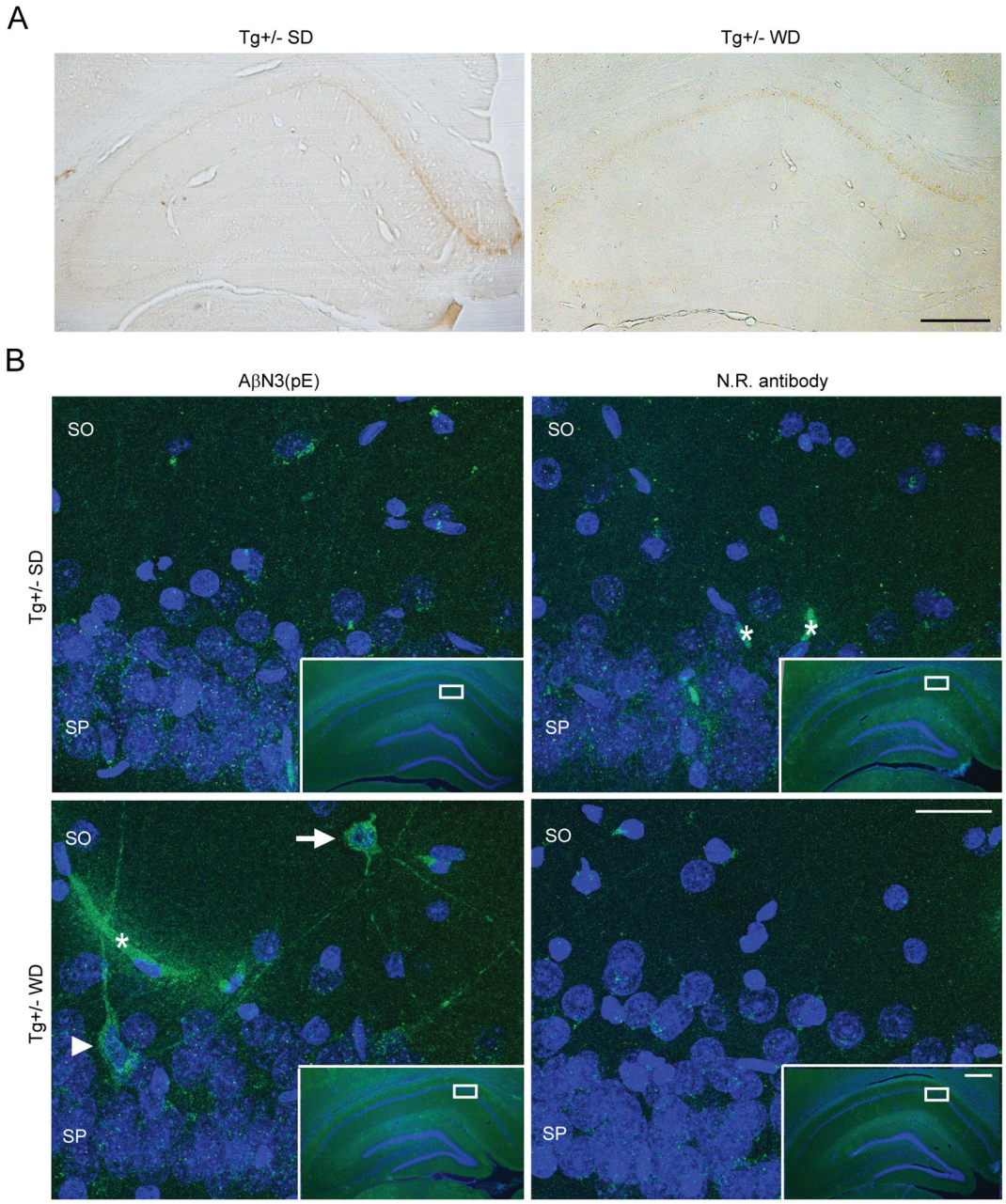

Fig. 6. Long-term Western diet causes intraneuronal accumulation of pathological AβN3(pE) aggregates in CA1 region of the hippocampus. (A), Representative microphotographs showing iAβ accumulation in hippocampus of Tg(+/−) rats, using an anti-Aβ(1−12) antibody (McSA1). Scale bar = 500 μm. (B), Representative microphotographs of CA1 hippocampal region from Tg(+/−) rats probed with an anti-AβN3(pE) antibody (left panels) or a non-related (N.R.) primary antibody (right panels). Arrowhead and arrow indicate AβN3(pE)-positive pyramidal neuron (green) in *stratum pyramidale* (SP) and AβN3(pE)-positive interneuron (green) in *stratum oriens* (SO), respectively, in the CA1 field; asterisk (*), non-specific label. Nuclei are stained with DAPI (blue). Insets, microphotographs of the whole hippocampus indicating the magnified CA1 area. Scale bar = 25 μm; inset, 500 μm. Tg(+/−)-SD, transgenic rats fed with standard diet (SD); Tg(+/−)-WD, transgenic rats fed with Western diet (WD); N.R. antibody, non-related primary antibody.

diet induces a marginal IR in the CNS that is only evident after a stimulation paradigm [2] and by in vitro experiments, demonstrating that in neuronal cultures AMPK/Akt signaling is only impaired if the extracellular glucose concentration is over 20 mM [52].

Our results also revealed that WD had no impact on cognition in WT rats. However, Tg(+/−) animals experienced aggravated learning and memory deficits after the exposure to WD, suggesting that this effect may be attributable to an interaction between diet biochemical and neuropathological status in this early AD model. The impact of a long-term use of a high-fat diet on cognitive performance in rodents remains controversial. While most of the reports show cognitive impairments [16,21,46,71,77,80], others show improvements [67] or no changes [3, 48]. Differences may be accounted by diet composition, time of exposure, age and gender of the animals and/or genetic differences between individual specimens.

Contrary to a recent report showing that peripheral IR alters lipid composition and function of hippocampal synapses in a transgenic mouse model of MetS [63], our results show unchanged hippocampal lipid composition in animals exposed to WD and impaired cognitive performance only in Tg(+/−). It is therefore important to revise the validity of the role of adipose tissue dysfunction-induced from peripheral organs to the brain as a mechanistic link between the worsened cognitive impairment and MetS. It was also proposed that the negative effects of high-fat diet on memory in AD-mouse models are associated with

early neuroinflammation while in non-Tg mice, neuroinflammation appears at advanced age [34]. High energy diet-induced inflammation in the rodent brain is a controversial topic with experimental evidence showing or not association [3,5,22,73]. Here we report that regardless of the genotype, feeding rats with a high-energy diet did not induce microglial activation, expression of inflammatory mediators or alteration of BBB permeability. In this regard, it seems that no comparable data could be obtained from different laboratories. However, after a careful analysis of bibliography we understand that in order to compare experimental data it is critical to know at least 4 parameters: the percentage of calories from fat (40% to 80%), the kind of fat (beef or lard), the carbohydrate supplementation (glucose or fructose) and the age of the animals. In support of this scenario, data have demonstrated that lard, but not beef fat, can disrupt cognition [83] and trigger glial activation [69] in rodents. Furthermore, it was suggested that brain aging is associated with a metabolic shift towards lipid/ketone body oxidation, which may be associated with inflammatory glial responses and cognitive decline [6,30]. Analysis of the inter-relationship in animal models among WD, age and AD-related neuroinflammation data suggests that a "basal neuroinflammatory tone" is required to be further enhanced by WD consumption. In this context, WD enhanced neuroinflamation in 3xTg-AD mice [34] that develop age-related, progressive neuropathology including plaques, tangle and glial activation but not in Tg(+/−) rats that do not develop neuroinflammation and plaque-like deposits across their lifespan.

In contrast to a report where high-fat diet induced decreased immunoreactivity of synaptophysin in a mouse model of accelerated aging [42], we did not find differences in the levels of synaptophysin or in the levels of other synaptic markers such as sintaxin-1 and PSD95. These results suggest that in WT and Tg(+/−) rats, WD per se is not enough to trigger an inflammatory phenotype and to alter synaptic integrity as opposed to the findings reported in different transgenic mouse models mimicking neurophatological features of late AD [10, 34,72], including senile plaques and gliosis.

We have previously reported that iAβ is associated with a chronic metabolic stress that impairs mitochondrial oxidative phosphorylation in synaptosomes isolated from Tg(+/−) hippocampus [41]. Here we show that WD induces significant decrements in the synaptosomal SRC and increments in the non-mitochondrial respiration regardless of the genotype, suggesting that a long-term exposure to high energy diet triggers a chronic metabolic stress that can damage mitochondria. SRC reflects the ability of mitochondria to meet increased energy demand with increased ATP production and it is considered a primary factor to define survival of neurons under stress [47]. In this regard, it was recently reported that in a context of mitochondrial dysfunction, neurons may attenuate endocytosis, which consumes most of the ATP at the synapses, in order to preserve ATP when needed [50]. Moreover, alterations in vesicle recycling have been linked to cognitive deficits [15]. The fact that WD cannot aggravate the SRC impairment in Tg(+/−) animals suggests that these animals have a lowered threshold for mitochondrial bioenergetics dysfunction that cannot be further downregulated. It is of note that synaptosomes from the hippocampus of WD-fed animals show significant increments in non-mitochondrial respiration compared to WT-SD that could be linked to an increase in reactive oxygen species formation. Remarkably, significant decrements were observed in the BHI of synaptosomes isolated from WD-treated rats suggesting that BHI could serve as a sensitive indicator of the capacity of synaptosomes to adapt their metabolic programs to withstand a challenging environment.

Sirt1 is highly expressed in hippocampal neurons and modulates synaptic plasticity and memory formation in the adult brain [20]. Brain-specific *SIRT1* knockout mice display cognitive impairment [44] and compelling evidence supports a major role of Sirt1 on neuronal plasticity, mitochondrial function and inflammatory pathways [45]. Here we show in Tg(+/−)-SD increments in Sirt1 transcript levels as compared to WT-SD. This result is in agreement with data reported from the inducible p25 transgenic mice, which show features of AD [33], and suggests that Sirt1 upregulation protects against neurodegeneration as previously reported [33]. In this context, it was shown that Sirt1 overexpression induces neurotrophic factors (BDNF, GDNF and VEGF) gene expression in mouse hippocampus [12] and can directly interact and regulate the activity of transcription factors (TFs) and coregulators critical for metabolism and ROS production, including PPAR-γ ([55]), PGC-1α [59], p53 [75] and the FOXO [7]. As a whole, our results may be interpreted as a neuroprotective adaptation response implying the role of Sirt1 as a stress sensor molecule during early stages of the disease. However, after exposure to long-term WD significant changes were observed in Sirt1 transcript levels in Tg(+/−)-WD as compared to Tg(+/−)-SD, indicating that WD represses hippocampal Sirt1 transcription. Similar results were observed in the hippocampus of mice fed with high-fat diets [25,81]. Transcriptional activity of SIRT1 is influenced by TFs acting on both the proximal and distal regions of the *SIRT1* promotor. While FOXOs, PPAR-α, PPAR-β, p21 and CREB activate *SIRT1* gene expression, p53, PPAR-γ, HIC1/CtBP act as negative regulators. It was proposed that excess of nutrients activates PPAR-γ which in turn binds to PPAR-γ response elements in the *SIRT1* promoter to repress *SIRT1* transcription [23,24]. Furthermore, Tg(+/−)-WD showed decreased expression of downstream *SIRT1* genes including *SOD2* and *PGC-1α* in accordance with reports in AD brains [57] and transgenic APP/PS1 mice exposed to high-fat diet [51]. It is interesting that the PPARs, which act as lipid sensors [66] control SIRT1 activity, with PPAR-γ, related to lipid anabolism, inhibiting *SIRT1* expression; whereas PPAR-α and PPAR-β/δ, both linked to fatty acid oxidation, increase Sirt1 mRNA levels.

The larger amount of nitrated proteins in Tg(+/−)-WD indicates that peroxynitrite, a highly oxidant specie derived from the reaction between $O_2^{\bullet-}$ and nitric oxide [58] is formed in this setting and that Tg(+/−)-WD brain is undergoing a sustained mitochondrial oxidative damage. These results are in agreement with previous reports showing that high-fat diet may affect the expression of genes involved in mitochondrial function and biogenesis [4,70] and that it increases 3-nitrotyrosine levels in the brain [77]. Since we did not find a global reduction in hippocampal CRTC1 protein levels in Tg(+/−)-WD rats, we could speculate that the reduction of Sirt1 mRNA levels did not impact on the impaired CRTC1-nuclear translocation reported in these animals [79], suggesting that WD worsens the cognitive performance by CRTC1-independent mechanisms. The alteration in the expression of genes related to cell energy control suggests that WD may also promote Aβ production by preventing the non-amyloidogenic processing of APP due to suppression of PGC-1α, as previously reported [57].

The impact of high-fat diet on APP processing was extensively analyzed in transgenic animal models of AD and the general consensus is that it aggravates cognitive performance and neuropathology by enhancing β-site cleavage of APP and potentiating Aβ accumulation in the brain [29]. In agreement with these observations, we detected increments in the APP-CTF fragments in the hippocampal homogenates from Tg(+/−)-WD rats compared to Tg(+/−)-SD and accumulation of Aβ38/40/42 species without changes in BACE1 transcript levels. These results suggest that high-fat diet-mediated increment of β-site cleavage might be caused by post-translational mechanisms that alter BACE1 protein stability and/or changes in the rate of BACE1 translation [11], or by regulation of subcellular trafficking of BACE1 [39], as previously reported. It is of note that hippocampal levels of Aβ40 and Aβ42 in Tg(+/−)-SD rats are 30- and 1500-fold lower, respectively, than values reported in AD brain of neuropathologically diagnosed cases [60]. As compared to transgenic mice, Aβ40 and Aβ42 brain levels in Tg(+/−) rat are similar or 100-fold lower depending on the transgenic model [10,29]. Moreover, the ratio Aβ40:Aβ42 is 5:1 in Tg(+/−) rats; 5:4 or 5:25 in transgenic mice showing Aβ immunostained plaque-like deposits and 5:50 in AD brains, suggesting the rat brain is very susceptible to the putative neurotoxic impact of Aβ42. The exacerbation of amyloidogenic APP processing by a nutritional challenge in rodents

was previously described [72]. In this study, we have observed 3-fold increments in Aβ40 and Aβ42 levels, without changes in Aβ40:Aβ42 ratio, in brains from Tg(+/−) rats fed with WD as compared to those fed with SD. Unchanged Aβ40:Aβ42 ratio after exposure of transgenic mice to long-term high-fat diet was also reported, suggesting that WD impacts on β-secretase and not on γ-secretase activity in accordance with previous reports [64]. In this study, increased activity of β-secretase in Tg(+/−)-WD brains is further supported by the accumulation of Aβ38 which was not detected in Tg(+/−)-SD brains. In contrast to published data showing that that dietary fat and/or sucrose intake accelerates AD-like pathogenesis in transgenic mice by reducing IDE levels which has been associated with IR [8,10,54,82], our results show that Aβ steady-state in the Tg(+/−)-WD brain is influenced by Aβ production but not Aβ catabolism mediated by IDE expression and/or activity. Furthermore, we found in Tg(+/−)-WD brains a restricted pattern of intracellular accumulation of AβN3(pE) in CA1 pyramidal neurons and interneurons in the SO, in accordance with its numerically minority in the hippocampus. However, interneurons exert a powerful control on network excitability through their widespread and strategically wired axon collateral systems [68]. Since all neurons in the SO are GABAergic [17], accumulation of AβN3(pE) in this layer could lead us to hypothesize that hippocampal neuronal excitability could be dysregulated in Tg(+/−)-WD rats as proposed in another animal model of AD [1]. It has been demonstrated that AβN3(pE) accumulates in the brain at the earliest stages of AD and may play an important role in the formation of pathological amyloid deposits [18,62]. In previous studies that employed well characterized Tg mouse models of AD, this peptide was not detected or, in some cases, was detected at a more advanced age [18,31,32,65]. Our results may be explained by the fact that rats are phylogenetically and physiologically closer to humans [76] and perhaps they could mimic better pathogenic processes relevant to human disease. To the best of our knowledge, this is the first study demonstrating the presence of N-truncated and post-translational modified Aβ peptide in a rodent brain at early age. In line with recent reports focusing on the importance of the composition of Aβ species [28] rather than their absolute amounts [35], our results shed light onto a specific pathological process occurring in early AD brain and triggered by a modifiable risk factor.

5. Conclusions

We propose a framework for a potential model in which energy-dense diet and early AD pathology affect systems which converge into intracellular pathways that regulate cognitive functions (Fig. 7). Our results show the harmful impact of unhealthy diet on the outcome of AD regarding Aβ accumulation and post-translationally modified Aβ species, nitroxidative stress, brain bioenergetics and cognitive function. This study supports the notion that dietary intervention aiming at optimizing fat and sugar consumption might be relevant for the prevention of AD, at least in people with genetic risk factors.

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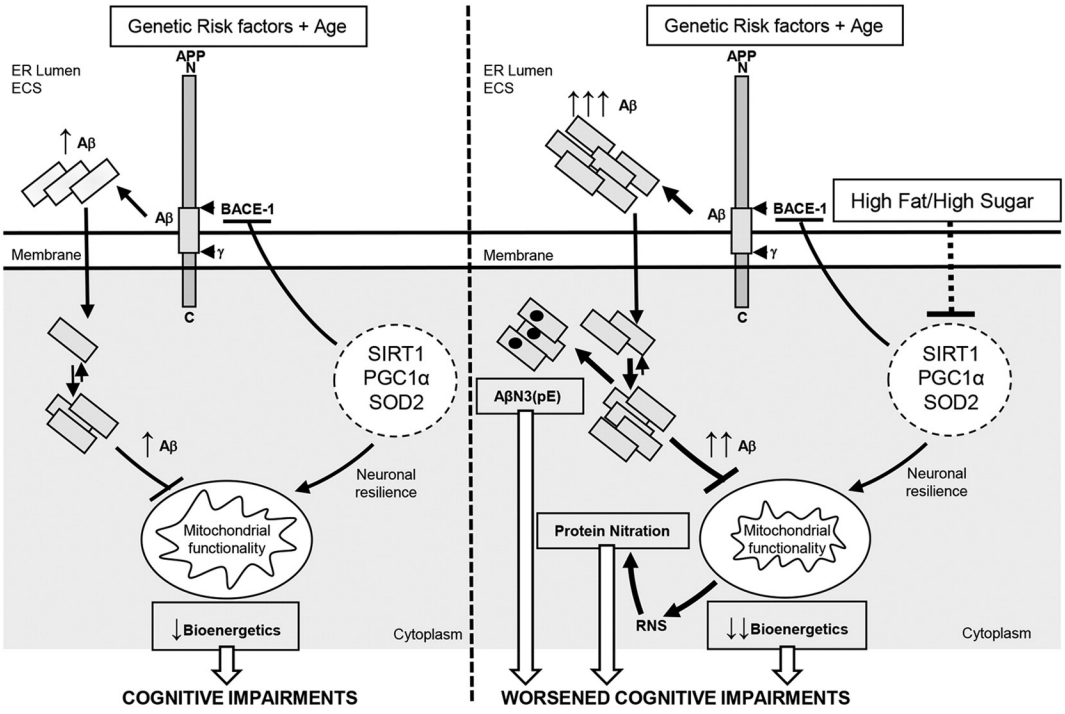

Fig. 7. Hypothetical mechanisms by which energy-dense diet (high fat/high sugar) aggravates the neuropathology in a transgenic rat model of early AD. Left panel, age and genetic risk factors promote accumulation of intracellular Aβ at early stages of AD. High levels of intracellular Aβ impair synaptic mitochondrial functionality, worsens bioenergetic deficits and cognitive impairments. This process is balanced by activation of neuronal resilience pathways (Sirt1, PGC-1α and SOD2) aimed to inhibit BACE-1 activity and ROS production. Right panel, energy-dense diet (high fat/high sugar) inhibits neuronal resilience pathways exacerbating Aβ production and bioenergetic deficits and promotes generation of AβN3(pE) neurotoxic isoform and protein nitration, aggravating cognitive impairments.

no roles in study design; data collection, analysis and interpretation of results.

Conflict of interests

The authors report no conflicts of interest.

Ethics approval

The study was carried out in strict accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) and the OLAW-NIH (Office Laboratory Animal Welfare) guidelines. The protocol was approved by the Animal Care Committee of Fundación Instituto Leloir (FIL) Assurance# A5168-01.

Author's contributions

All authors read and approved the final manuscript. PVMA performed biochemical, bioenergetics and histological analysis, interpreted data and helped to draft the manuscript and figures; PG performed behavioral tests, histological analysis, ELISA tests, statistics analyses and helped to draft the manuscript; MLW elaborated the high-energy diet supplement; NO, LIB and CRT designed the nutritional protocol; CQ assisted PVMA in bioenergetics experiments and in the interpretation of the results; AR analyzed lipid composition in the brain; ESP and CD determined peripheral metabolic parameters; SDC, RR, GG and ACC supplied regents critical for the study; EMC collaborated in the interpretation of the data and in the draft of the manuscript; LM oversaw the experimental design, data analysis, data interpretation, and drafting/editing the manuscript.

Transparency document

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2016.12.014.

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