Redox Biology 75 (2024) 103242

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Glucose 6 phosphate dehydrogenase overexpression rescues the loss of cognition in the double transgenic APP/PS1 mouse model of Alzheimer's disease

Angela G. Correas [a,1], Gloria Olaso-Gonzalez [a,*,1], Marta Roca [b], Mari Carmen Blanco-Gandía [c], Carla Nascimento [a], Agustin Lahoz [b,d], Marta Rodriguez-Arias [e], José Miñarro [e], Mari Carmen Gomez-Cabrera [a,2], José Viña [a,2]

[a] Freshage Research Group, Department of Physiology. Faculty of Medicine, University of Valencia, CIBERFES, Fundación Investigación Hospital Clínico Universitario/ INCLIVA, Valencia, Spain
[b] Analytical Unit, Instituto de Investigación Sanitaria Fundación Hospital La Fe, Valencia, Spain
[c] Departamento de Psicología y Sociología, Facultad de Ciencias Sociales y Humanas, Universidad de Zaragoza, Teruel, Spain
[d] Biomarkers and Precision Medicine Unit, Instituto de Investigación Sanitaria Fundación Hospital La Fe, Valencia, Spain
[e] Unidad de Investigacion Psicobiologia de las Drogodependencias, Departamento de Psicobiologia, Facultad de Psicologia, Universidad de Valencia, Valencia, Spain

ARTICLE INFO

Keywords:
Alzheimer's disease
G6PD
Oxidative stress
Glucose metabolism
Amyloid-β
Pentose phosphate pathway

ABSTRACT

Mice models of Alzheimer's disease (APP/PS1) typically experience cognitive decline with age. G6PD overexpressing mice (G6PD-Tg) exhibit better protection from age-associated functional decline including improvements in metabolic and muscle functions as well as reduced frailty compared to their wild-type counterparts. Importantly G6PD-Tg mice show diminished accumulation of DNA oxidation in the brain at different ages in both males and females. To further explore the potential benefits of modulating the G6PD activity in neurodegenerative diseases, triple transgenic mice (3xTg G6PD) were generated, overexpressing APP, PSEN1, and G6PD genes. The cognitive decline characteristic of APP/PS1 mice was prevented in 3xTg G6PD mice, despite similar amyloid-β (Aβ) levels in the hippocampus. This challenges the dominant hypothesis in Alzheimer's disease (AD) etiology and the majority of therapeutic efforts in the field, based on the notion that Aβ is pivotal in cognitive preservation.

Notably, the antioxidant properties of G6PD led to a decrease in oxidative stress parameters, such as improved GSH/GSSG and GSH/CysSSG ratios, without major changes in oxidative damage markers. Additionally, metabolic changes in 3xTg G6PD mice increased brain energy status, countering the hypometabolism observed in Alzheimer's models. Remarkably, a higher respiratory exchange ratio suggested increased carbohydrate utilization. The relative failures of Aβ-targeted clinical trials have raised significant skepticism on the amyloid cascade hypothesis and whether the development of Alzheimer's drugs has followed the correct path. Our findings highlight the significance of targeting glucose-metabolizing enzymes rather than solely focusing on Aβ in Alzheimer's research, advocating for a deeper exploration of glucose metabolism's role in cognitive preservation.

1. Introduction

Alzheimer's disease (AD) pathophysiology is extremely complex and heterogeneous, involving the accumulation of senile plaques caused by abnormal amyloid-β (Aβ) metabolism and the accumulation of neurofibrillary tangles caused by tau hyperphosphorylation. There is also an increase in the levels of reactive oxygen species (ROS) which induces the transcription of pro-inflammatory genes and the release of cytokines and chemokines that cause neuroinflammation [1]. Neuronal energy metabolism is also compromised in this disease. In particular, there is a decline in glucose metabolism which is one of the earliest and most common abnormalities observed in AD patients [2]. The Pentose

* Corresponding author.
E-mail address: gloria.olaso@uv.es (G. Olaso-Gonzalez).
[1] These authors contributed equally to this work and these senior authors
[2] also contributed equally to the work.

https://doi.org/10.1016/j.redox.2024.103242
Received 2 April 2024; Received in revised form 13 June 2024; Accepted 15 June 2024
Available online 17 June 2024

Phosphate Pathway (PPP) provides pentose availability to DNA and RNA synthesis, involves NADPH formation which confers protection against oxidative stress, and is an alternative to obtain glycolytic intermediaries such as glyceraldehyde-3-phosphate. Glucose-6-phosphate dehydrogenase, G6PD, is the enzyme at the crossover between glycolysis and the PPP [3].

The G6PD enzyme is present in a wide range of tissues in mammals, with the greatest concentration found in immune cells and testes [4]. While mice that lack the G6PD enzyme do not survive, hypomorphic G6PD alleles are common in humans, affecting about 5 % of the global population, and approximately 400 million people in the world [5].

G6PD overexpressing mice (G6PD-Tg) are partially protected from some age-associated declines: they have improved glucose tolerance and insulin sensitivity, an increased median lifespan in females [6], and old-G6PD overexpressing mice are less frail than their WT controls [7]. Importantly G6PD-Tg mice show diminished accumulation of DNA oxidation (measured as 8-hydroxyguanosine) in the brain and other tissues such as the liver at different ages in both males and females [6]. The fact that oxidative stress is associated with AD [1,8] prompted us to generate a triple transgenic mouse that overexpresses APP/PS1 and G6PD. The APP/PS1 mice are a double transgenic mice model that has been genetically engineered to express a hybrid mouse/human amyloid precursor protein called Mo/HuAPP695swe, as well as a mutated form of human presenilin 1 known as PS1-dE9, in their central nervous system neurons. These mutations have been linked to the development of an AD phenotype at an early age. We hypothesized that the antioxidant properties of G6PD could alleviate the pathological signs of the experimental disease.

Our results show that the overexpression of G6PD rescues the cognitive decline caused by AD pathology in the 2xTg mice (APP/PS1). This protective effect is based on a dual mechanism targeting two hallmarks of AD: oxidative stress and the brain's energy deficit.

To the best of our knowledge, this is the first time that overexpressing an enzyme involved in general carbohydrate metabolism shows a clear protective effect in an experimental AD model.

2. Material and methods

Generation of triple transgenic (APP/PS-1/G6PD) mice. We crossed heterozygous B6C3-Tg(APPswe, PSEN1dE9)85Dbo/Mmjax, from now on (2xTg) mice with homozygous C57BL6/J-OlaHsd Tg-G6PD (G6PD-Tg) [6]. The animals generated were, thus, triple transgenic for the APP, PSEN1, and G6PD genes from now on (3xTg G6PD), or transgenic only for G6PD. The 3xTg G6PD was used for further experiments. B6C3 wild-type (WT) mice were also used as controls. All the determinations were performed when mice were 12- to 14-month-old because the transgenic mice develop Aβ deposits in the brain by 6–7 months of age so both mutations are associated with early-onset Alzheimer's disease. We used both male and female mice. We did not see any difference between sexes in any of the parameters analyzed as shown in the supplementary materials (see Figs. S1–S3)

All the animals used in this study were raised and housed in the animal facility of the Faculty of Medicine of the University of Valencia under temperature conditions of 22 ± 2 °C and 55 ± 5 % humidity. All the experimental protocols have been approved by the Animal Ethics Committee of the University of Valencia (protocol numbers: A1549115230055 and A1549125701978).

Functional assessment. Mice from the three groups were functionally evaluated at an age of 12.6 ± 0.5 months. Grip strength, motor coordination, and a fatigue resistance test were performed in these animals. Briefly, to assess grip strength a Grip Strength Meter (Panlab, Harvard Apparatus) consisting of a T-bar connected to a dynamometer was used. Mice were restrained so that they could grasp the bar with their forelimbs and the maximum force was recorded in three attempts [9].To assess motor coordination, the Rotarod Test (Panlab model LE8205, Harvard Apparatus) was performed as previously described [10]. An incremental treadmill test was performed using a treadmill (Panlab model LE8710MTS, Harvard Apparatus) with a 5 % incline to assess fatigue resistance. The test was performed starting with a speed of 10 cm/s for the first 4 min, increasing the speed by 4 cm/s every 2 min. The time it takes for the mice to reach fatigue and the speed reached were recorded [11].

Cognitive assessment. The passive avoidance test was used to evaluate the learning and memory of the mice. A box with a compartment illuminated with a lamp and another in darkness separated by a gate is used. On the first day of the test or training, the mouse is placed in the lighted area with the door closed. After 60 s, the gate is opened and the time it takes for the mouse to move into the dark zone is recorded, at which point it is shocked at 0.5 mA for 3 s, and then the animal is taken back to its cage. The test is carried out 24 h after training, in which the mouse is placed back in the illuminated compartment, recording the time it takes to pass into the dark area; if it has not crossed after 300 s, the test is terminated [12–14]. The test was performed again at 7 days to assess implicit long-term memory.

The Hebb–Williams maze was used to assess the learning ability and spatial memory of mice. It consists of a battery of mazes in which the time it takes the mice to find the exit is computed, such that a longer time required to complete them is associated with deterioration in cognitive status. Cold water (about 15 °C) is used as a stimulus to motivate the animal to look for the exit, where dry paper is placed. The test is carried out in 8 days, in each of which a different maze is used, increasing its difficulty or complexity. The first three days of the test are training or habituation days, in which the animal freely explores the environment, and no water is used. For each trial, the maximum time allowed to complete the test is 300 s; once that time has elapsed, the animal is taken out of the maze regardless of whether it has managed to find the exit, and the test is considered finished [15–17].

In vivo metabolic assessment. Respiratory metabolism was assessed by indirect calorimetry with the OxyletPro System (Panlab, Harvard Apparatus) in 13- to 14-month-old 2xTg (n = 5) and 3xTg G6PD (n = 11) mice. They were single-housed with ad libitum access to food and water and maintained at 20–22 °C under a 12:12 h light: dark cycle (light period 08.00–20.00). Oxygen consumption was determined by measuring oxygen concentration in air entering the chamber compared with air leaving the chamber. Measurement in each chamber was recorded for a total of 48 h to obtain the parameters of VO_2, VCO_2, energy expenditure, and respiratory quotient, calculated as VCO_2/VO_2. For the analysis of the data, the mean value of each of the parameters was calculated at each hour, and they were represented as a function of time.

Glucose 6-P dehydrogenase activity. G6PD activity was determined spectrophotometrically by measuring the absorbance at 340 nm after the addition of NADP as described previously [18] in cortex samples of 14-month-old WT (n = 12), 2xTg (n = 11) and 3xTg G6PD mice.

Amyloid-β **levels determination.** Hippocampal Aβ levels were measured by an Aβ 1–42 enzyme-linked immunosorbent assay (ELISA) with reference KHB3441 (ThermoFisher). Sample preparation, processing, and detection were performed according to the manufacturer's instructions.

Carbonylated proteins levels. Immunoblot detection of protein carbonyl groups in cortex samples was assessed using the 'Oxyblot Protein Oxidation Detection Kit' (Millipore, USA). Briefly, 20 μg of total protein was loaded into gels before electrophoretic separation and transfer onto PVDF membranes. Total protein carbonyls were quantified as densitometry of the blotting divided by the total density of the ponceau red staining. Specific proteins were visualized by enhanced chemiluminescence using a BioRad scanning densitometer and quantified with ImageJ software.

Lipid peroxidation determination by HPLC. Lipid peroxidation was determined in cortex samples as described previously [19]. Briefly, this method is based on the hydrolysis of lipid peroxides and subsequent formation of the adduct thiobarbituric acid (TBA) and malondialdehyde,

MDA (TBA-MDA2). This adduct was detected by reverse phase HPLC and quantified at 532 nm. The chromatographic technique was performed in an isocratic mobile phase that is a mixture of 50 mM KH2PO4 (pH 6.8) and acetonitrile (70:30).

Total Glutathione levels. Total GSH was determined as previously described [20]. Briefly, the method is based on the catalytic action of GSH or GSSG in the reduction of Ellman reagent (DTNB) by a mixture of TPNH and glutathione reductase. The procedure measures the total glutathione (GSH + GSSG) content of unknown mixtures and is not subject to appreciable interference by the presence of other thiol components.

Metabolomic analysis. Samples were prepared following a standard protocol for polar metabolites that is further described elsewhere [21]. For the analysis of cortex samples from 2xTg and 3xTg G6PD mice, liquid chromatography equipment coupled to a high-resolution mass spectrometer with an Orbitrap UPLC-QExactive Plus detector was used. The chromatographic and mass spectrometry conditions were the following: UPLC column: Xbridge BEH amide 2.5 μM (2.1*150 mm) Waters, Chromatogram time: 25 min, Vinj: 5 μL, Column temperature: 25 °C, Autosampler temperature 4 °C, Flow: 105 μL/min, Mobile phase A = H_2O 10 mM. Orbitrap parameters, Mode: ESI pos and neg, Event 1 *m/z* range: 70–700Da, Event 2, Range *m/z*:700–1700Da, Full Scan Resolution: 140,000, AGC:3e6, Maximum IT: 200 ms centroid acquisition. Metabolite feature extraction was carried out using the EI-Maven platform [22]. Metabolite identification was performed using an in-house library based on MS/MS spectra and internal standards, which ensures the highest level of metabolite identification following the recommendations of the Metabolomic SocietySumner et al., 2007[23]. Once annotated, the data sets were analyzed with MetaboAnalyst 5.0 software, including a Pathway enrichment analysis [24].

Citrate synthase activity. Frozen cortex samples were homogenized with Tris 75 mM, EDTA 2 Mm and 0.1 % Triton-X (pH 7.4) buffer. The homogenates were sonicated and centrifuged at 1.500g and the supernatant were diluted and used for total protein and activity determination. Spectrophotometric cuvettes were used with 881.7 μL of previous buffer, 3.3 μL of acetyl-CoA (10 mg/mL), 100 μL of DTNB (1 mM), 10 μL of sample and 5 μL of oxalacetate (50 mM) for absorbance measuring during 5 min at 420 nm. Enzymatic activity results were relativized by amount of total protein, determined with Lowry method.

Western blot. Homogenates of mice cortex samples were prepared using Tris 75 mM, 2 % SDS and 10 % glycerol (pH 7.4) containing phosphatases and proteases inhibitor cocktail, to which and Laemmli Buffer was added. Proteins were separated in SDS-polyacrylamide in gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Primary antibodies against VDAC (1:1000, Cell Signaling, ref. 4866) and against cytochrome C (1:1000, Santa Cruz, ref. sc-13156) were used.

Statistical methods. Values are expressed as the mean ± standard deviation (SD). The normal distribution of the samples was assessed by the Shapiro–Wilk or Kolmogorov-Smirnov tests. To compare two different groups, the unpaired Student's t-test was used, or the Wilcoxon test in case of a non-normal distribution. A volcano plot was also employed to show statistical significance (p-value) versus magnitude of change (fold change) when comparing the two groups. When comparing three different groups a one-way analysis of variance (ANOVA) or the Kruskal-Wallis analysis for non-normal distribution data was used. In the case of studying two independent variables, a two-way ANOVA test was used.

3. Results

3.1. G6PD overexpression rescues the cognitive impairment in Alzheimer's mice

To assess cognitive function in mice and to evaluate their memory and learning, we employed two distinct tests: the passive avoidance and the Hebb-Williams tests. Within the Hebb-Williams tests, two mazes were utilized: Maze 1, an easy one, and Maze 5, a difficult one. A visual representation of both assessments is presented in Fig. 1A.

Fig. 1B shows the results of the passive avoidance test. Our findings show that 2xTg mice exhibit the capacity to learn and avoid the deleterious region of the maze 24 h after the training period. However, a week later, these mice exhibited diminished implicit long-term memory, indicative of a tendency to "forget" the associated danger in the test (the shock). On the contrary, the healthy controls (WT) and the 3xTg G6PD mice consistently recall the imperative avoidance behavior thus showing a higher memory retention.

Panels C and D show the outcomes of the Hebb-Williams test, depicting the time taken by mice to navigate either Maze 1 or Maze 5. The disparities between 3xTg G6PD and 2xTg mice are statistically significant, with the latter displaying inferior outcomes. Notably, these differences are more prominent when confronted with higher difficulty levels, particularly in Maze 5, as highlighted in panel E.

Panel F elucidates the cumulative cognitive performance, measured as areas under the curve, for the three mouse groups in both Maze 1 and 5. The data indicate that the cognitive decline observed in 2xTg mice is ameliorated by the overexpression of G6PD. This underscores the pivotal role of G6PD in preserving cognitive function in the face of cognitive challenges in our model.

3.2. G6PD overexpression does not decrease the hippocampal Aβ levels

In elucidating the observed alterations in cognitive function, we analyzed hippocampal Aβ levels. Our results reveal a marked increase in Aβ content in Alzheimer's transgenic mice compared to WT controls, as illustrated in Fig. 2A. It is noteworthy that WT mice, even in advanced life stages, do not exhibit Aβ plaques. The Aβ levels in the brains of 3xTg G6PD animals were found not to be statistically different from those in 2xTg mice. This seemingly paradoxical result, significant cognitive effects in the absence of discernible changes in Aβ levels, suggests that Aβ may not be a pivotal factor in maintaining cognition, at least in this experimental model. The lack of direct correlation between Aβ levels and cognitive decline has been also reported in preclinical as well as clinical studies [25–29]

Furthermore, we investigated the G6PD activity in the cortex across the various mouse cohorts. Notably, the G6PD activity in the WT and 2xTg mice did not exhibit a statistically significant difference. In contrast, the 3xTg G6PD mice, displayed a significantly elevated activity of this enzyme, as depicted in Fig. 2B.

3.3. Enhanced physical performance in G6PD overexpressing mice: comprehensive evaluation of body weight, grip strength, endurance capacity, and motor coordination

In our assessment of various physical parameters, including body weight (Fig. 3A), grip strength (Fig. 3B), endurance capacity (Fig. 3C), and motor coordination (Fig. 3D), across all experimental groups, a consistent trend emerges. The overexpression of G6PD in the 2xTg mice consistently leads to improved physical performance in the animals. These findings align with our prior investigations involving ordinary mice that solely overexpressed G6PD, revealing a protective effect against age-associated functional decline and frailty [7]. The collective data underscore the potential benefits of G6PD overexpression in promoting overall physical well-being and performance in murine models.

3.4. Assessing redox status in 2xTg mice. Insights into G6PD-mediated antioxidant effects

Given the well-established association between advanced AD and oxidative stress [30], we examined the redox status in the 3xTg G6PD mice, comparing it with WT and 2xTg counterparts.

In cortex samples we measured oxidative stress parameters, GSH/GSSG and GSH/CysSSG, as well as markers of oxidative damage, MDA,

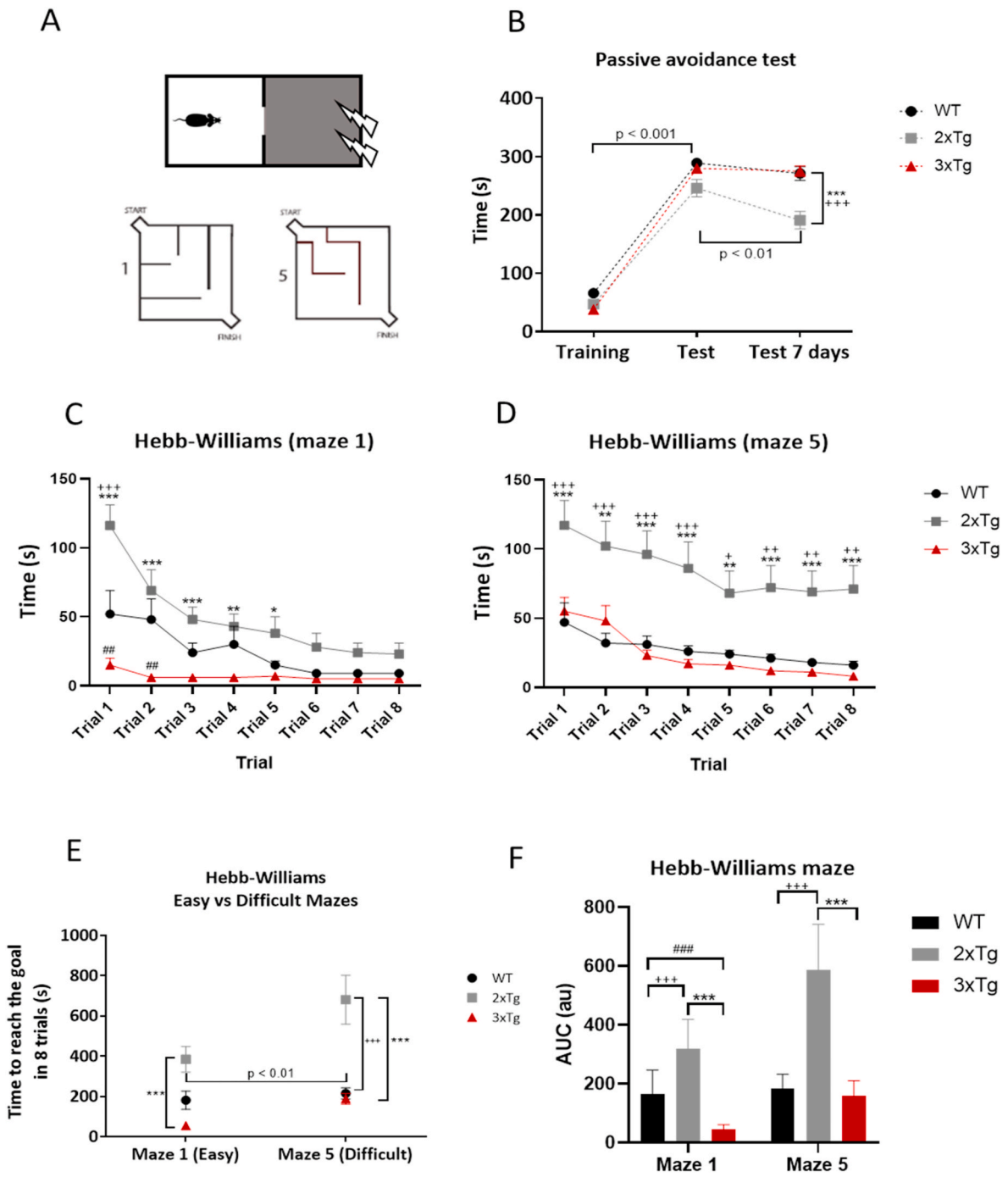


Fig. 1. G6PD overexpression reduces cognitive impairment in 2xTg mice. A) Cognitive tests design: Passive avoidance test (up), Hebb-Williams Maze 1 (left down), and Hebb-Williams Maze 5 (right down). **B)** Passive avoidance test results. N = 22–26 mice/group. ***p < 0.001 (3xTg G6PD vs 2xTg), $^{+++}$ p < 0.001 (2xTg vs WT). **C)** Hebb-Williams easy maze (maze 1) results. N = 22–26 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001 (3xTg G6PD vs 2xTg), $^{+++}$ p < 0.001 (2xTg vs WT), $^{\#\#}$p < 0.01 (3xTg G6PD vs WT). **D)** Hebb-Williams difficult maze (maze 5) results. N = 22–26 mice/group. **p < 0.01, ***p < 0.001 (3xTg G6PD vs 2xTg),+p< 0.05, $^{++}$ p<0.01, $^{+++}$ p < 0.001 (2xTg vs WT). **E)** Hebb-Williams Easy vs Difficult Mazes results. N = 22–26 mice/group. ***p < 0.001 (3xTg G6PD vs 2xTg), $^{+++}$ p < 0.001 (2xTg vs WT). Hebb-Williams mazes area under the curve. ***p < 0.001 (3xTg G6PD vs 2xTg), $^{+++}$ p < 0.001 (2xTg vs WT), $^{\#\#\#}$p < 0.001 (3xTg G6PD vs WT).

and protein carbonyls. Our analysis revealed changes in oxidative stress but not in oxidative damage markers between the different experimental groups.

While the total glutathione levels in the brain cortex remained unchanged in both 2xTg and 3xTg G6PD mice, the glutathione/redox ratios showed that the 3xTg G6PD mice exhibited a less oxidized state in the cortex, when compared to their 2xTg counterparts. These results, shown in Fig. 4 (panels C, D, and E), underscore the potential antioxidant effects of G6PD overexpression, offering a glimpse into its role in modulating the redox equilibrium in the context of AD.

However, we did not find a significant increase in a marker of lipid peroxidation or protein carbonylation in either 2xTg or 3xTg G6PD mice, as illustrated in Fig. 4, panels A and B. These findings align with our prior observations that oxidative damage typically manifests in advanced stages of the disease [31], suggesting a nuanced temporal relationship between oxidative stress and Alzheimer's progression.

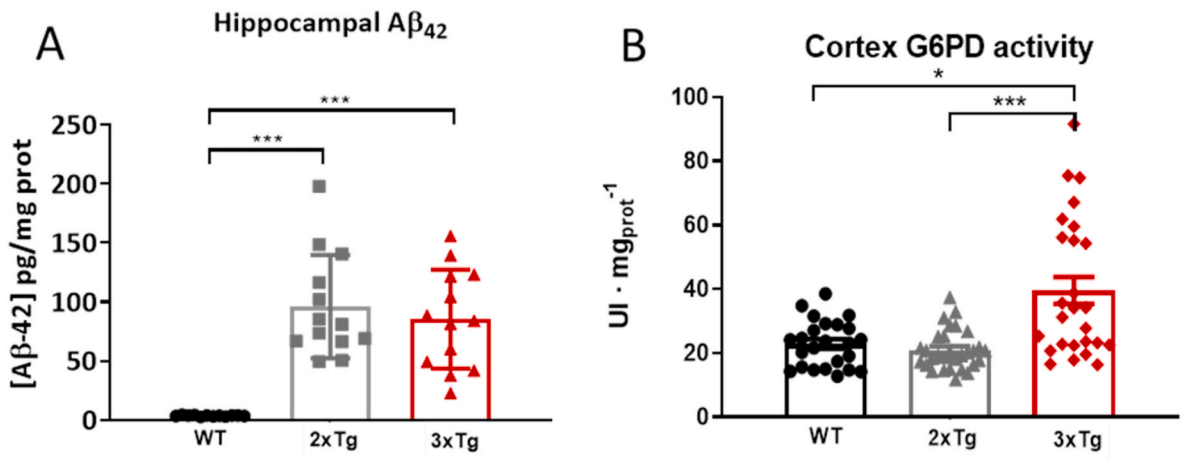


Fig. 2. Cortical G6PD activity and Aβ42 hippocampal levels in WT, 3 Tg-G6PD, and 2xTg mice. A) Hippocampal Aβ42 levels ***p < 0.001. N = 13–14 mice/group **B)** Cortex G6PD activity. ***p < 0.001. N = 24–30 mice/group.

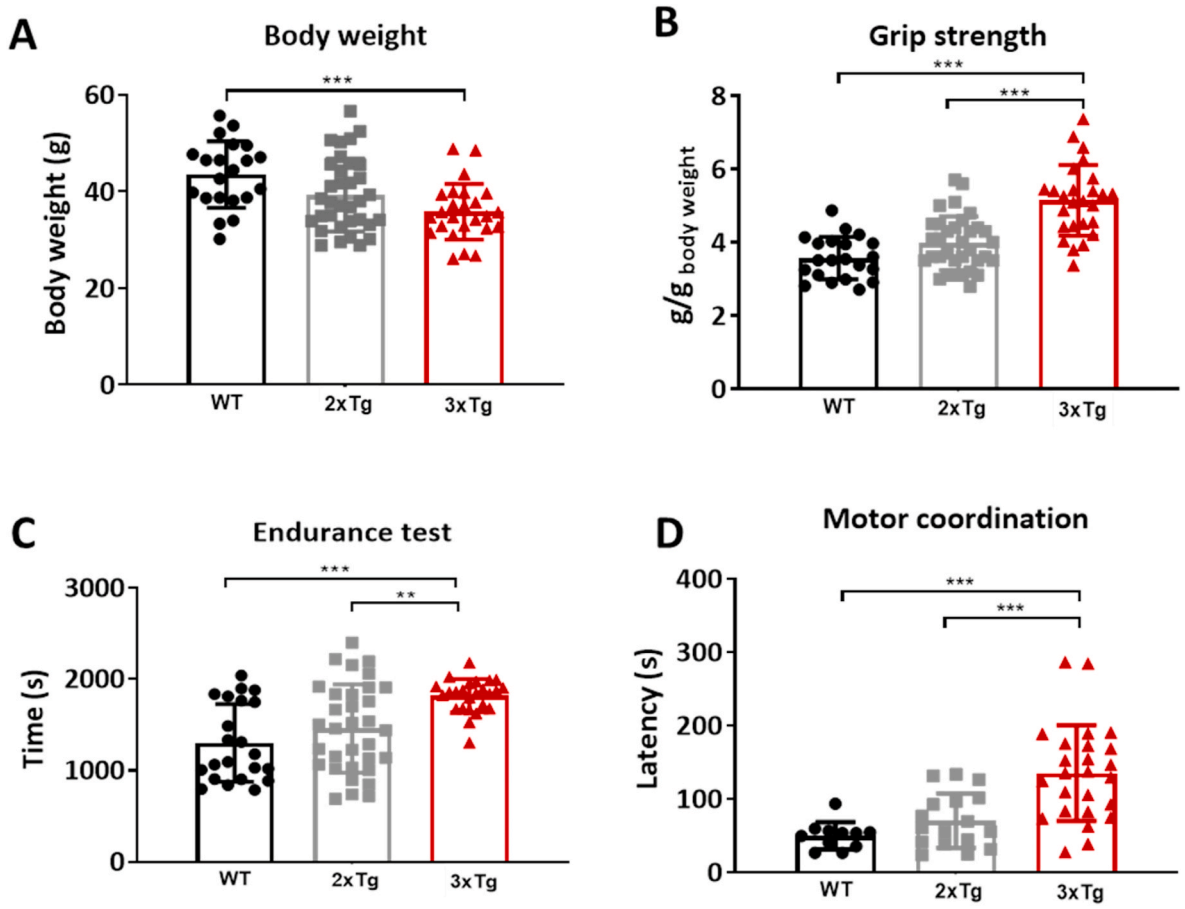


Fig. 3. 3xTg G6PD mice exhibit an improved physical performance when compared with WT and 2xTg mice. A) Body weight. **B)** Grip Strenght. **C)** Aerobic Resistance. **D)** Motor coordination. N = 11–35 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001.

3.5. G6PD overexpression in 2xTg mice modulates energy expenditure and substrate utilization

G6PD serves a crucial role by not only generating reducing equivalents in the form of NADPH but also redirecting glucose toward pentose production rather than pyruvate synthesis. To unravel the metabolic impact of G6PD overexpression in the 2xTg mice, we conducted *in vivo* experiments, evaluating whole-body energy expenditure (EE) and the respiratory exchange ratio (RER), defined as the ratio of CO_2 production to O_2 utilization.

Fig. 5 shows that the EE and RER values were lower during the light vs dark cycles in all the animals independently of the genotype. The measurements recorded across the two light and dark cycles were averaged by analyzing the total area under the curve. Panels A and B

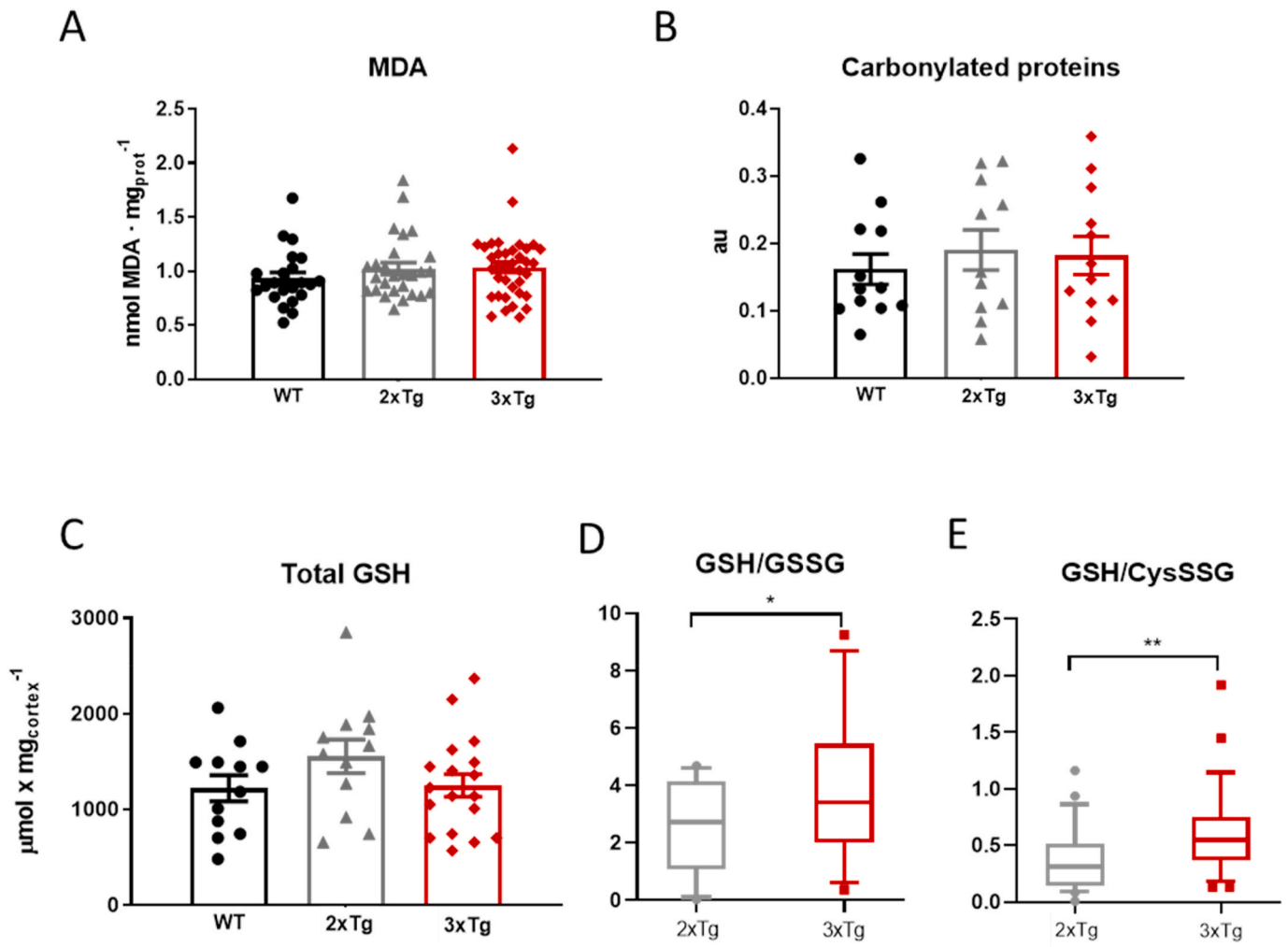


Fig. 4. Cortical oxidative stress and oxidative damage markers in WT, 2xTg, and 3xTg G6PD animals. A) MDA levels. N = 22–37 mice/group **B)** Carbonylated proteins levels. N = 12 mice/group **C)** Total GSH. N = 12–18 mice/group **D)** GSH/GSSG ratio measured from metabolomic determinations. N = 25–27 mice/group **E)** GSH/CysSSG ratio measured from metabolomic determinations. N = 25–27 mice/group * $p < 0.05$, **$p < 0.01$.

show that G6PD overexpression leads to a significant reduction in energy expenditure among Alzheimer's mice. This effect, quantified in Panel 5B, is estimated to be approximately 5 % of the value observed in the 2xTg mice without the G6PD overexpression.

During the light cycle the 3xTg G6PD mice consistently exhibit a substantial increase in RER, approaching values around 0.9, as depicted in Fig. 5C. In contrast, 2xTg animals maintain values around 0.75, indicative of a predominant reliance on lipid consumption. The fact that the 3xTg G6PD mice display a RER closer to one indicates a heightened utilization of carbohydrates as the primary fuel source [32].

3.6. Metabolic changes associated with overexpression of G6PD in Alzheimer's mice

Fig. 6 shows a metabolomic analysis of cortex samples from both 2xTg and 3xTg G6PD animals. The Volcano plot in Fig. 6A reveals significant elevations in five key metabolites—phosphocreatine, ATP, acetyl CoA, GTP, and 2-hydroxy glutarate—in the 3xTg G6PD when compared with 2xTg. These metabolites, are connected to the Krebs cycle, signifying an augmented availability of carbon units for the cycle and an enrichment of high-energy phosphate-containing molecules, as exemplified in Panel 6B.

These findings collectively indicate heightened glucose utilization and an enhanced energy status in the brains of Alzheimer's mice overexpressing G6PD (3xTg G6PD). The documented hypometabolism in the brains of Alzheimer's mice seems to be counteracted by G6PD overexpression, as elucidated in Panel 6C, showcasing the improved energy charge of the ATP/ADP system.[33]

Additionally, we have measured three mitochondrial content markers in cortex samples from WT, 2xTg and 3xTg G6PD mice: VDAC levels (Panel 6D) and CytC levels (Panel 6E), both measured by Western Blotting, and citrate synthase activity (Panel 6F) measured using an enzymatic method. As can be seen, 2xTg mice have a higher level of VDAC than WT and 3xTg G6PD and there is no change in the levels of CytC or citrate synthase activity.

Furthermore, our exploration into redox dynamics reveals a noteworthy shift in the NAD^+/NADH (Fig. 6G). The 3xTg G6PD mice exhibit a significantly decreased NAD^+/NADH ratio compared to 2xTg mice. This change, although not accompanied by a change in the NADP+/NADPH ratio (Fig. 6H), underscores the multifaceted impact of G6PD overexpression in orchestrating an augmented energy utilization and an improved redox balance in the brains of the AD mice.

4. Discussion

4.1. Overexpression of G6PD restores cognition in the 2xTg mice model of AD

Current therapeutic strategies for AD-related dementia primarily target two of the neuropathologic hallmarks of the disease,

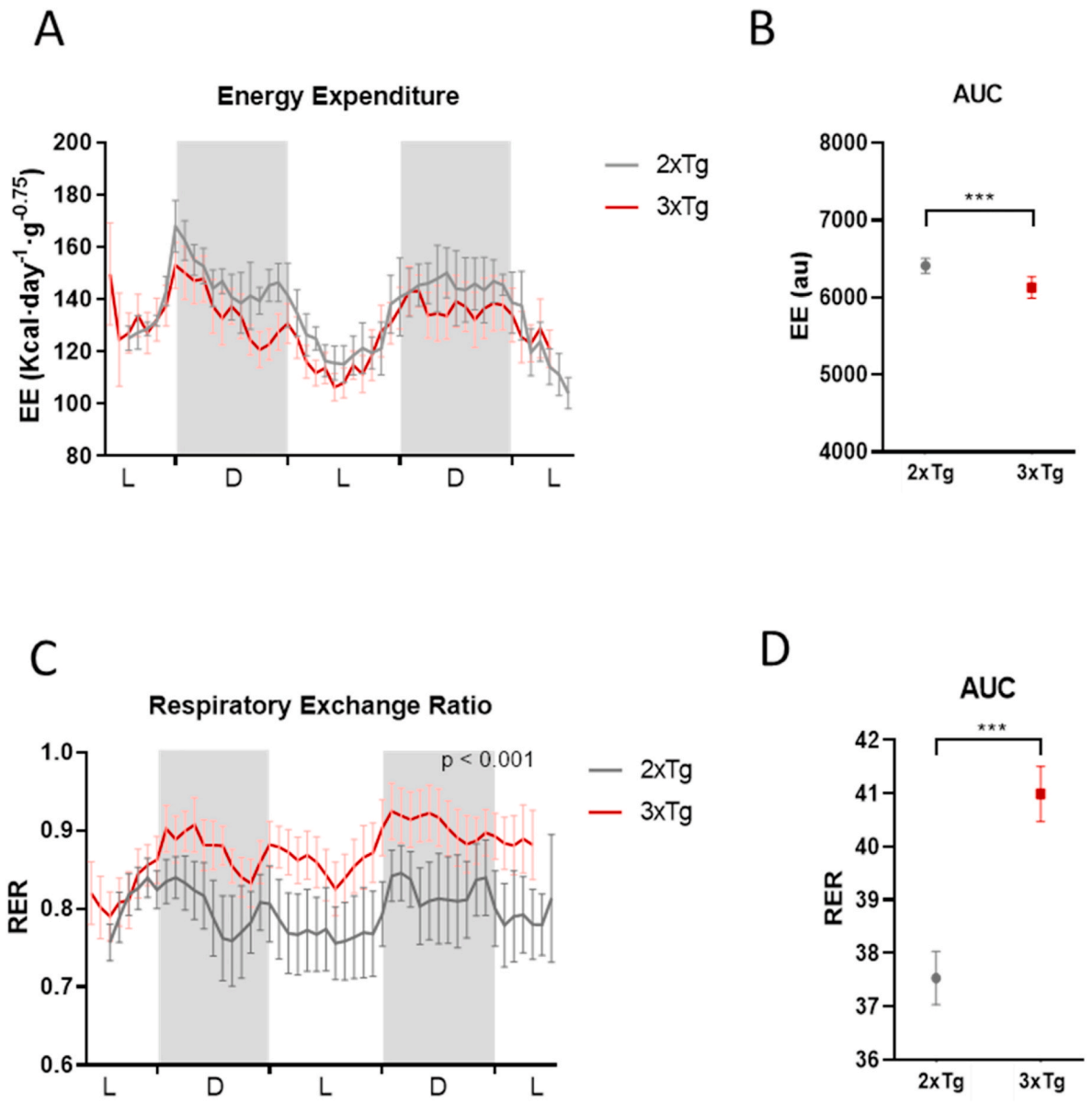


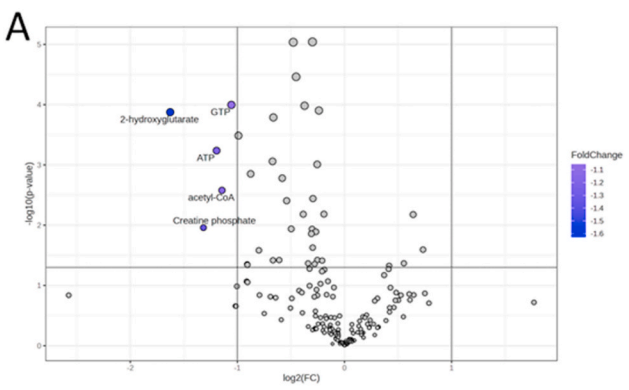
Fig. 5. 3xTg G6PD mice show an enhanced glucose metabolism. A) Energy expenditure (EE) over 48 h (N = 5–11 mice/group), **B)** Area under the curve. **C)** Respiratory exchange ratio over 48h (RER) (N = 5–11 mice/group). **D)** Area under the RER curve. (N = 5–11 mice/group). ***p < 0.001.

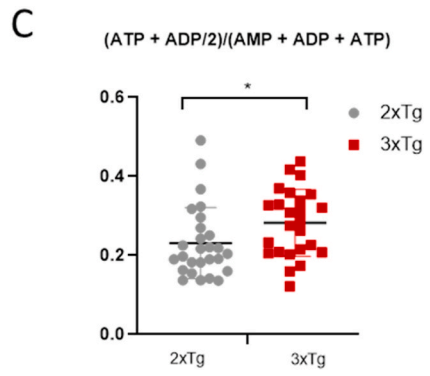
hyperphosphorylated tau as neurofibrillary tangles and Aβ deposition as neuritic plaques [34]. The most commonly used treatments for Alzheimer's disease belong to the family of cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists [35]. In the United States, Lecanemab and Aducanumab are two approved monoclonal antibodies targeting amyloid for treating AD. These antibodies represent the initial disease-modifying therapies for AD. However, a deeper understanding of the metabolic processes in Alzheimer's brains reveals specific changes related to glucose metabolism that extend beyond neuron loss [36,37]. In fact, glycolytic alterations in AD patients have been previously reported, including a decreased activity of phosphofructokinase due to Aβ binding [36]. Brain function relies mainly on glucose as an energy substrate. Primary concept of brain metabolism suggests that astrocytes use glucose and transform it into lactate to support the energetically demanding neuronal processes responsible for plasticity and memory formation. Bolaños and colleagues underscored the different glucose metabolic pathways, with neurons favouring the PPP and glial cells exhibiting a preference for glycolysis [38].

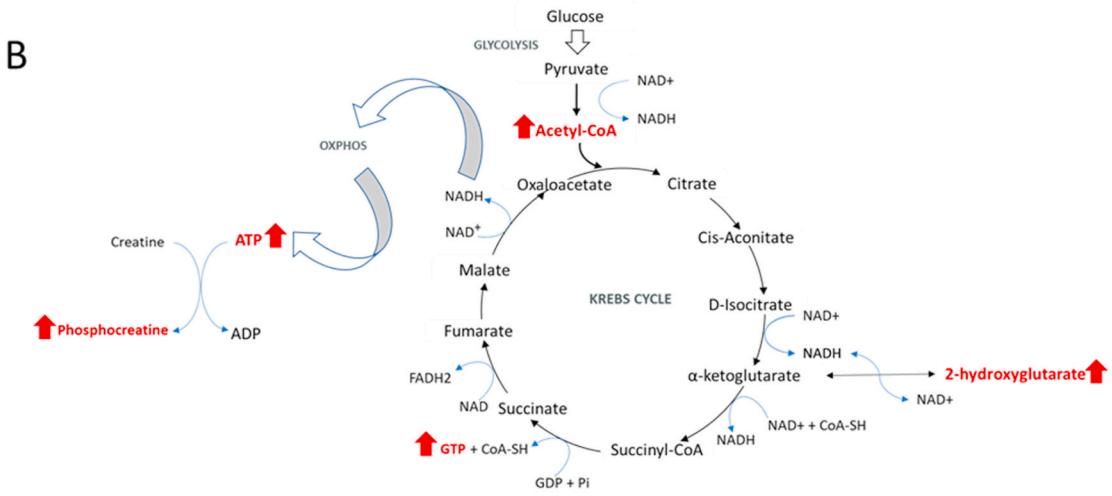
Back in 2016, we generated the G6PD transgenic mice [6]. These mice displayed ubiquitous 2-fold overexpression of G6PD mRNA, protein and activity in most tissues [6]. G6PD-Tg mice presented improved health parameters as they aged: enhanced glucose tolerance and insulin sensitivity, better neuromuscular coordination, and less weight gain with age. We associated the improved health span of G6PD-Tg mice to a decrease in age-associated oxidative damage to macromolecules. In recent years, substantial data have accumulated, suggesting that the brain in AD patients shows a redox imbalance, potentially contributing to the progression of neuron degeneration and subsequent cell death in this disorder. Therefore, in this study, we examined the hypothesis that overexpression of G6PD could potentially mitigate cognitive decline in the 2xTg mouse model of Alzheimer's disease.

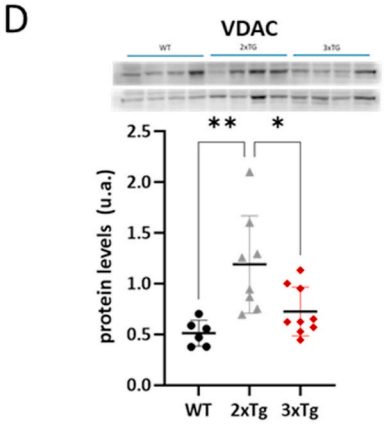
After the evaluation of implicit long-term memory, learning ability and spatial memory of mice, here we report that G6PD overexpression reverses cognitive impairment in middle-age conventional APP/PS1 Alzheimer's mice (2xTg).

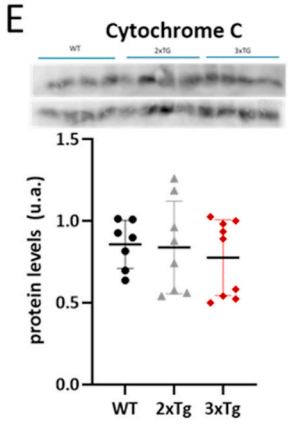
Our approach targets a specific enzyme acting on a glucose catabolic pathway, offering a novel perspective on AD treatment. Previous studies focused on enzymes involved in Aβ or p-tau production [12]. This is the

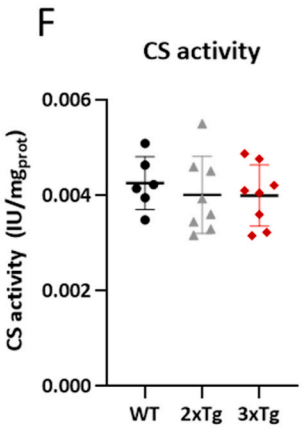


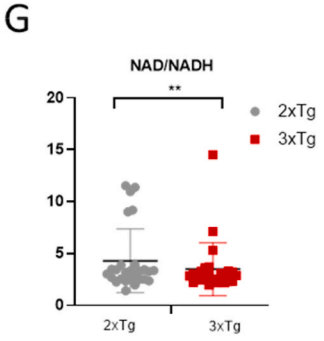












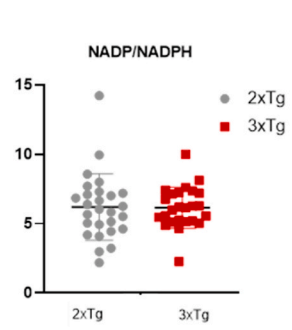


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Fig. 6. 3xTg G6PD mice show a boost in brain energy metabolism. A) Volcano plot shows, in blue, the metabolites found at different levels (fold-change>2, $p < 0.05$) in 3xTg G6PD vs 2xTg mice cortex samples. Blue dots represent the metabolites found at different levels for the comparation of 3xTg G6PD vs 2xTg mice cortex samples (fold-change >2, $p < 0.05$. **B)** Representation of the main pathways in which the metabolites selected are involved. C) Adenylate Energy Charge Ratio in 3xTg G6PD and 2xTg mice cortex samples. D) VDAC levels in WT, 2xTg and 3xTg G6PD mice cortex. E) CytC levels in WT, 2xTg and 3xTg G6PD mice cortex samples. F) Citrate synthase activity in WT, 2xTg and 3xTg G6PD mice cortex samples. G) NAD^+/NADH ratio in 3xTg G6PD and 2xTg mice cortex samples. H) $NADP^+$/NADPH ratio in 3xTg G6PD and 2xTg mice cortex samples. (N = 24–26 mice/group). *$p < 0.01$, **$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

first instance where overexpressing a glucose-consuming enzyme improves cognition in experimental animals. Correcting metabolic dysregulation in the Alzheimer's brain emerges as a promising avenue to impede disease progression [39].

The multifaceted neuroprotective role of G6PD and the PPP in adult brain injury and neurodegenerative diseases, as highlighted by numerous studies [40–45], extends to protecting dopaminergic nigrostriatal neurons from parkinsonism through G6PD overexpression [46]. This emphasizes the potential therapeutic impact of G6PD modulation in addressing various neurodegenerative conditions.

4.2. Redox-mediated mechanisms underlie the beneficial effects of G6PD overexpression in the 2xTg mice model of AD

G6PD plays a role in counteracting the oxidative stress inherent in aerobic life by utilizing glucose to generate a continuous flow of reducing equivalents.

Recent research has shed light on G6PD's significance in neurological and neurodegenerative disorders, showcasing its neuroprotective potential against oxidative damage, such as that observed in cerebral ischemia [45]. Studies on post-mortem brain samples from Alzheimer's patients revealed an increase in G6PD activity in specific brain regions, including the hippocampus and cerebellum [47]. Similarly, mouse models of Alzheimer's disease displayed elevated G6PD activity in the brain [48,49], with high levels of G6PD activity in the serum suggesting diagnostic potential for early Alzheimer's detection [50].

Previous work from our group showed reductive stress in early AD patients [31]. This is in keeping with an increased activity of G6PD in early phases of AD. Thus, a compensatory increase in G6PD may be a mechanism to lower radical-mediated damage.

While G6PD overexpression in our study effectively reduced oxidative stress in the 2xTg AD mice model, there were no changes in oxidative damage markers (MDA for lipids and carbonyls for proteins). Notably, we found a significant increase in the GSH/GSSG and GSH/CSSG ratios, indicative of protection against oxidative stress, but no changes in oxidative damage. Oxidative stress is related to reversible alterations of the cellular compartments redox-status that may affect redox pairs such as the GSH/GSSG or the GSH/CSSG. However, oxidative stress is not necessarily always accompanied with oxidative damage. The latter cause irreversible damage to cellular molecules (like DNA oxidation, lipid peroxidation, or protein carbonylation). When the redox state of the cell becomes more reduced, oxidation of thiols leading to disulfides can be reversed, on many occasions catalyzed by specific enzymes such as glutathione reductase. Thus, it is considered that the oxidative stress markers are reversible [51]. Oxidative damage, however, is irreversible unless there are specific repair mechanisms like DNA repair systems or proteasomal degradation for the oxidized proteins.

In general, it is considered that oxidative stress precedes oxidative damage which occurs when reducing systems cannot cope with the rate of the oxidation of cell components. Thus, our results may reflect that in this study we are dealing with a prodromal phase of the disease in the 2xTg mice model. Although the animals show a significant increase in their Aβ levels and in markers of oxidative stress, they do not yet show an increase in markers of brain oxidative damage.

In concordance with a recent study [52], our results reveal G6PD's potential in protecting against oxidant-mediated neurodegenerative disorders.

4.3. G6PD overexpression activates brain energy metabolism in the 2xTg AD animal model

The comparison between 3xTg G6PD and 2xTg mice demonstrates elevated levels of key energy-related metabolites (ATP, GTP, and phosphocreatine) and Krebs cycle-associated metabolites with G6PD overexpression.

A landmark study in 1979 by Bowen and colleagues revealed a profound alteration in glycolysis regulation, specifically the inhibition of phosphofructokinase (a regulatory enzyme of the glycolytic pathway), contributing to low glucose consumption in the brains of Alzheimer's patients [37]. This inhibition poses a significant hurdle to maintaining glycolytic activity in the affected brain regions. The PPP bypasses phosphofructokinase blockade in Alzheimer's brain and hence improves glucose energy metabolism in AD brain.

We hypothesize that the maintenance of cognition in 3xTg G6PD mice is maybe explained by G6PD's ability to overcome the blockade imposed by fructose 6 phosphate dehydrogenase inhibition in Alzheimer's, ensuring a more adequate energy supply. This dual role of G6PD, acting as an antioxidant and influencing energy metabolism, positions it as a promising target for therapeutic interventions in AD.

The PPP, renowned for producing reducing equivalents (NADPH) and pentoses for nucleic acid synthesis, emerges as a key player in preserving glycolytic activity under conditions of phosphofructokinase inhibition, a characteristic observed in AD. Notably, the PPP generates 3-phosphoglyceraldehyde, a metabolite easily convertible to pyruvate even in aging animals. G6PD overexpression enhances the carbon flow toward pyruvate and subsequently acetyl CoA, facilitating entry into the Krebs cycle. This unique attribute of the PPP, particularly when phosphofructokinase is inhibited, offers a plausible explanation for the observed improvements in metabolic status and the energy index in our G6PD-overexpressing Alzheimer's mice.

Metabolomic signatures have previously revealed significant disturbances in energy metabolism in the 2xTg mice model, mainly in the hippocampus and the cortex [53].

Decreased brain levels of lactic acid, malic acid, creatinine, 2-hydroxyglutaric acid, citric acid, and glucose-6-phosphate have been previously reported [53]. The decrease of brain glycolytic intermediates levels supports a reduced carbohydrate metabolism, while reduced citrate and malate could be behind the perturbed Krebs cycle, in agreement with previous studies [54]. The deficiency of 2-hydroxyglutarate points to disrupted mitochondrial activity in the brain of the 2xTg mice given that this compound is a by-product resulting from a side reaction of malate dehydrogenase [55]. Our results show a significant increase in the cortical levels of this metabolite in the 3xTg G6PD mice (see Fig. 6). Furthermore, altered homeostasis of the phosphocreatine system also occurs in the brain in AD mice models. A significant decrease of creatine (Cr) in all the brain areas in the 2xTg mice has been previously reported. Moreover, recent studies using AD mouse models have shown that supplementation with Cr improves brain bioenergetics, as well as AD biomarkers and cognition [56]. Interestingly, our results also show a significant increase of this metabolite in the brain of the 3xTg G6PD mice (see Fig. 6). The brain Cr system plays a crucial role in maintaining bioenergetic flux. Phosphocreatine, synthesized from Cr and ATP in the mitochondria is used to regenerate ATP in the cytosol by the mitochondrial and cytosolic isozymes of creatine kinase, respectively, thus helping to maintain ATP homeostasis. This reasoning fits perfectly with the increases in ATP levels detected in the brains of 3xTg G6PD mice in

the metabolomic analysis (See Fig. 6). Finally, increased levels of acetyl-CoA in different brain areas result in neuroprotection and increased acetylation of histone H3K9 in SAMP8 mice, a site linked to memory enhancement [57]. In our study, we have found an increase in the acetyl-coA in the 3xTg G6PD mice brains when compared to the 2xTg model (See Fig. 6).

We have determined that 2xTg mice had a higher level of VDAC than 3xTg G6PD mice, although the former have a worse brain energetic status (see Fig. 6). It has been previously described that VDAC1 levels are elevated in the affected regions of AD postmortem brains and cortical tissues from APP transgenic mice [58–60], which can be a compensatory response to the mitochondrial dysfunction that Aβ may induce [61].

Overall, our results show that defects in energy metabolism are a key hallmark in the APP/PS1 transgenic mice of AD, involving multiple metabolic pathways such as glycolysis, TCA cycle, oxidative phosphorylation or phosphocreatine system. Overexpression of G6PD, the rate limiting enzyme in the PPP pathway, activates energy metabolism in the 2xTg mice. In Fig. 6, Panel C, we illustrate a significant increase in the brain's energy state in the 3xTg G6PD mice compared to the 2xTg mice.

4.4. G6PD beyond antioxidant defense. Expanding its role in health and disease, with implications for Alzheimer's prevention

Long acknowledged as a key antioxidant enzyme in erythrocytes, G6PD's deficiency has been historically associated with health conditions like neonatal jaundice, hemolytic anemia, and favism. Recent studies, however, have broadened our understanding of G6PD, linking its deficiency to increased susceptibility to viral infections and degenerative diseases [62,63].

The findings presented in this paper, alongside our prior research demonstrating G6PD's role in delaying frailty onset, have prompted us to explore avenues for elevating this enzyme in humans. Lifestyle modifications, particularly increased controlled exercise, have emerged as effective strategies for boosting G6PD levels, as shown in our previous work [7]. Conversely, G6PD deficiency, the most prevalent human mutation evolved for protection against malaria, poses challenges in later life—a classic example of antagonistic pleiotropy, as termed by Sir Peter Medawar [64].

The practical implications of these insights suggest a proactive approach to identify lifestyle changes or, if feasible, specific treatments that elevate G6PD activity. This becomes particularly relevant for individuals at risk of developing Alzheimer's, such as those with a family history of the disease.

5. Conclusions

The relevant facts reported here are that G6PD overexpression reverses the cognitive deficits in the APP/PS1 model of AD, and that this may be due to combined favourable effects on NADPH production and on increased energy metabolism.

Funding

This work was supported by the following grants: Instituto de Salud Carlos III CB16/10/00435 (CIBERFES); (PID2022-142470OB-I00) from the Spanish Ministry of Innovation and Science; PROMETEO (CIPROM/2022/56) de "Consellería de Educación, Universidades, y Empleo de la Generalitat Valenciana"; EU Funded H2020- DIABFRAIL-LATAM (Ref: 825546); Red EXERNET-RED DE EJERCICIO FISICO Y SALUD (RED2022-134800-T) Agencia Estatal de Investigacion (Ministerio de Ciencias e Innovación); Ramón Areces Foundation. Part of the equipment employed in this work has been funded by Generalitat Valenciana and co-financed with ERDF funds (OP ERDF of Comunitat Valenciana 2014–2020).

CRediT authorship contribution statement

Angela G. Correas: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Gloria Olaso-Gonzalez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marta Roca:** Visualization, Validation, Methodology, Investigation. **Mari Carmen Blanco-Gandía:** Methodology, Investigation, Data curation. **Carla Nascimento:** Methodology, Investigation. **Agustin Lahoz:** Formal analysis, Conceptualization. **Marta Rodriguez-Arias:** Investigation, Formal analysis, Conceptualization. **José Miñarro:** Supervision, Investigation, Conceptualization. **Mari Carmen Gomez-Cabrera:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **José Viña:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT4 in order to improve language and readability, with caution. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103242.

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