Resveratrol induces brain resilience against Alzheimer neurodegeneration through proteostasis enhancement

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Abstract

Resveratrol is a natural compound that mimics the antioxidant and antiaging effects of caloric restriction, mainly mediated through SIRT1, a deacetylase that induces longevity and neuroprotection. We aimed to analyze the effects of resveratrol on the brain status of control non-transgenic (NoTg) and AD transgenic (3xTg-AD) mice to discern the mechanisms involved in a potential inducement of resilience against age-related neurodegeneration and Alzheimer's disease (AD). Mice were fed with a diet supplemented with 100 mg/kg of *trans*-resveratrol from 2 months of age during 10 months. Resveratrol administration induced complete protection against memory loss and brain pathology in 3xTg-AD mice, and also induced cognitive enhancement in healthy NoTg mice. Resveratrol improved exploration and reduced anxiety in both mouse strains, indicative of well-being. Resveratrol reduced the presence of Aβ and p-tau pathology in the hippocampus of the 3xTg-AD mouse. Proteostasis analysis showed the following in both NoTg and 3xTg-AD mice: (i) increased levels of the amyloid degrading enzyme neprilysin; (ii) reduction of the amyloidogenic secretase BACE1, and (iii) increase of proteasome protein levels and enhancement of proteasome activity. Resveratrol also increased AMPK protein levels, then upregulating the SIRT1 pathway, as shown by the activation of PGC-1α and CREB in both mice, resulting in further beneficial changes. Our data demonstrated that resveratrol induces cognitive enhancement and neuroprotection against amyloid and tau pathologies. Improvement of proteostasis by resveratrol, in both healthy and AD mice, suggests that it is a mechanism of brain resilience and defense against neurodegeneration caused by the accumulation of aberrant proteins.

Keywords

Resveratrol, SIRT1, proteasome, neuroprotection, 3xTg-AD

Abbreviations

Aβ, amyloid-β; AD, Alzheimer's disease; ADAM10, a disintegrin and metalloproteinase 10; ANOVA, analysis of variance; AMPK, adenosine monophosphate-activated protein kinase; p-AMPK, phosphorylated AMPK; APP, amyloid precursor protein; APP-CTF, C-terminal fragment of APP; bw, body weight; BACE1, beta-site APP cleaving enzyme 1; BPSD, behavioral and psychological symptoms of dementia; CHIP, carboxyl-terminus of Hsp70 interacting protein; CREB, cAMP response element-binding protein; p-CREB, phosphorylated CREB; CSF, cerebrospinal fluid; Hsp70, heat shock protein 70; IDE, insulin-degrading enzyme; MWM, Morris water maze; NAD^+, nicotinamide-adenine dinucleotide; NOR, novel object recognition; NoTg, control non-transgenic mice; ac-p53, acetylated p53; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PSD95, postsynaptic density protein 95; ac-tau, acetylated tau; p-tau, hyperphosphorylated tau; UPS, ubiquitin proteasome system.

Introduction

The progressive increase in life expectancy has led to an increase in the incidence of age-related diseases, including dementia [1]. Alzheimer's disease (AD) is the most common cause of dementia in the elderly [2,3], characterized by brain depositions of amyloid-β (Aβ) and hyperphosphorylated tau (p-tau), leading to synapse dysfunction, cognitive and memory deficits and, finally death [4,5]. To date, there is no effective treatment of AD, except for temporarily symptom-relieving drugs [6,7]. Finding a treatment is crucial to reducing the overall effects of aging, increasing healthspan in humans.

Resveratrol is a polyphenol found in common dietary sources such as grapes, berries, peanuts and red wine, and in some herbal remedies [8,9]. In animal models, resveratrol exhibits a wide spectrum of potential therapeutic activities, including antioxidant, anti-inflammatory, neuroprotective and longevity-promoting properties [9-11]. Experimental studies suggest that resveratrol is active against AD pathogenesis [12-15]. First clinical trials of dietary supplementation with resveratrol in AD have been completed, with encouraging changes such as attenuation of the decline of cerebrospinal fluid (CSF) levels of Aβ species [16,17], and reduction of plasma levels of pro-inflammatory markers and attenuation of cognitive and functional decline [17]. Furthermore, improvement of cognitive performance reported in trials with non-demented older adults [18,19] suggests a preventive potential of resveratrol. Studies with transgenic mouse models of AD showed that resveratrol intake protected against Aβ plaque formation in Tg19959 [20] and APP/PS1 mice [21,22]. Increased synaptic markers and preservation of recognition memory were also found in resveratrol treated APP/PS1 mice [22]. Moreover, in the p25 mouse model of AD and tauopathies, intracerebroventricular delivery of resveratrol prevented impairment of fear conditioning associative learning and reduced the levels of markers of apoptosis and astrogliosis [23].

The hypothesis of the most widely accepted mechanism comprises that resveratrol mimics the antioxidant and antiaging effects of caloric restriction [24,25], which are mediated by SIRT1 [22,26]. SIRT1 is a nicotinamide-adenine dinucleotide (NAD^+)-dependent deacetylase associated with anti-aging pathways [27] that induces protective effects against AD brain pathology through regulating the acetylation homeostasis of key proteins [28-30]. There is controversy over whether resveratrol may be a direct activator of SIRT1 [31] or whether SIRT1 is indirectly activated by other resveratrol-induced pathways [32,33]. Recent evidences suggest that resveratrol increases adenosine monophosphate-activated protein kinase (AMPK) activity, leading to an increase of NAD^+ levels, which in turn enhances SIRT1 activity [34,35].

At the cellular level, resveratrol demonstrated protective effects against oxidative stress and inflammatory processes induced by Aβ in PC12 cell line [36] and human stem cells [37]. Resveratrol promotes Aβ clearance through enhancement of proteasome-dependent proteolysis, as shown in cell lines expressing APP695, either wild-type or harboring the Swedish mutation [38] and in a *C.elegans* model of AD [39]. Resveratrol was also shown reducing Aβ levels of transgenic cell line and worm models by autophagy and lysosomal degradation activated by AMPK signaling [21,39]. Furthermore, resveratrol may decrease Aβ generation by favoring the non-amyloidogenic pathway of APP degradation [26].

One of the molecular changes of aging that might contribute to the development of AD is the deficiency in cellular control mechanisms that degrade aberrant proteins [40]. Clearance of Aβ and tau through proteolytic mechanisms include ubiquitin-proteasome system (UPS), autophagy-lysosomal system, and extracellular proteases [41]. Furthermore, protein folding stress in the endoplasmic reticulum may activate the unfolded protein response aimed to restore proteostasis, preferentially through autophagy in the AD brain [42], or trigger apoptosis of irreversible damaged cells [43]. However, the stress responsivity of the different AD mouse models is highly variable [44]. UPS is the

primary selective mechanism to maintain proteostasis in eukaryotic cells and is involved in many nerve cell functions, such as plasticity and memory [45,46]. Increasing evidence postulates functional alterations of UPS and its molecular components as causes of early changes in AD pathology [47]. Heat shock protein 70 (Hsp70) facilitates the ubiquitination of aberrant proteins through interaction with the carboxyl-terminus of Hsp70 interacting protein (CHIP) and the E3 ligase [48]. Polyubiquitinated proteins are recognized by the proteasome complex for subsequent proteolytic degradation by the 20S catalytic core [49,50].

Studies with AD mouse models were needed to confirm resveratrol-induced cognitive improvement and further unveil its mechanism of action against AD-like neurodegeneration. We aimed to analyze the effects of the administration of resveratrol in mice as a preventive and therapeutic agent, with emphasis in APP processing and UPS activity, and their effects on learning and memory. For this purpose, we treated both control non-transgenic mice (NoTg) and triple-transgenic mice for AD (3xTg-AD) with a daily dose of 100 mg/kg of resveratrol during 10 months. Our results demonstrated that resveratrol administration induced complete protection against memory loss and brain pathology in AD mice. Furthermore, we showed that resveratrol induced proteostasis enhancement in both 3xTg-AD and healthy NoTg mice. We propose that proteostasis enhancement increases brain resilience against neurodegeneration. New insights into the mechanisms of resveratrol in preclinical studies may aid in the design of preventive strategies against AD.

Materials and methods

Animals

Male 3xTg-AD mouse strain harboring familial AD mutations of the APP (APP_{Swe}) and the Presenilin 1 ($PS1_{M146V}$), and a tau gene mutation (Tau_{P301L}) [51] was used in the present study. These mice mimic many of the critical hallmarks of AD as Aβ and tau pathologies, impaired learning and memory, presence of behavioral and psychological symptoms of dementia (BPSD)-like, and oxidative stress [30,52]. Furthermore, 3xTg-AD mice reproduce the temporal course and areas affected by amyloid and tau pathology of AD neuropathology [53]. Control NoTg mice had the same genetic background hybrid 129 × C57BL/6 than 3xTg-AD mice [51]. Genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies. Animals were individually housed in Makrolon® cages under standard laboratory conditions of food and water *ad libitum*, 22 ± 2°C, and 12h:12h light-dark cycle. Animal breeding, treatment, and behavioral studies were performed at the University of Barcelona Animal House (UB, Barcelona, Spain). Animal handling and experimental procedures were approved by the Ethics Committee for animal experimentation (CEEA) of the University of Barcelona (UB) (Ref: DAAM 6523, CEEA), in accordance with the Decree 214/1997 of the Generalitat of Catalonia and the Directive 2010/63/EU of the European Union for animal experiments.

Resveratrol administration

At 2 months of age, mouse standard diet (2018 Teklad Global 18 % Protein Rodent Maintenance Diet, Harlan) was supplemented with 1 g/kg of *trans*-resveratrol (Mega Resveratrol, Candlewood Stars, Inc., CT, USA). Resveratrol groups (RV) received 100 mg/kg bw/day during 10 months. The period of 2 to 12 months of age covers a broad period of the AD pathology progression in 3xTg-AD mice, from the pre-symptomatic to the advanced pathology phase. Control groups (Ct) received standard diet. The experimental groups were as follows: NoTg-Ct (n = 14), NoTg-RV (n = 12), 3xTg-Ct (n = 10) y 3xTg-RV (n = 10). No significant differences were found among the treatment groups in diet intake or in body weight along the study (not shown).

Behavioral and cognitive tests

Animals were tested for behavior and cognitive improvement at 10 months of the chronic resveratrol treatment, at 12 months of age. The behavioral tests were carried out at the Unitat d'Experimentació Animal of the Faculty of Psychology of the University of Barcelona (Campus Mundet, UB). Selected BPSD-like symptoms and cognitive tests were analyzed as previously described [54,55]. Briefly, the *Open field* test was used to evaluate vertical and horizontal locomotor activity and general behavior in a white chamber during 5 min. The *Boissier's 4 hole-board* test was utilized to evaluate exploratory behavior by measuring head-dipping during 5 min. The *Dark & Light* test was employed to assess anxiety during 5 min in a black compartment connected to a lit compartment. The *Novel object recognition (*NOR) test was used to evaluate recognition memory, and is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The animals were submitted to a 10 min acquisition trial in the presence of two identical novel objects (A1 + A2). A 10 min retention trial occurred 2 h later, replacing object A1 with object B; and another 10 min retention trial took place 24 h later, replacing object A2 with object C. Discrimination index was calculated as [novel (t) – familiar (t)] / [total time (t) at novel + familiar]. The *Morris water maze* (MWM) test was employed to assess spatial learning and memory, and consisted of 1 day of cue learning, 6 days of learning acquisition, and 1 final day of memory retrieval. Animals were trained to locate the hidden platform in a circular water tank by relying on distinctive landmarks as visual cues (four trial sessions of 60 sec per day). On the last day, the

platform was removed and the mice performed a 60 sec probe trial to test learning retention. A computerized tracking system (SMART, Panlab S.A., Barcelona, Spain) was employed to measure escape latency, and distances and quadrants covered. At the end of the behavioral tests, the animals were decapitated under light anesthesia and the hippocampus and cerebral cortex were dissected and stored at −80°C for further analysis.

Western blotting

Protein extracts from hippocampus and cerebral cortex were obtained in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 10 µg/mL aprotinin. Aliquots of 30 µg of protein were analyzed for Western blot analysis by standard procedures [30,56]. The following antibodies were employed for immunodetection: Aβ clone 6e10, sAPPα, sAPPβ, C-terminal fragment of APP (APP-CTF), a disintegrin and metalloproteinase 10 (ADAM10), AMPK, phosphorylated AMPK (p-AMPK), beta-site APP cleaving enzyme 1 (BACE1), cAMP response element-binding protein (CREB), phosphorylated CREB (p-CREB), Hsp70, IDE, neprilysin, acetylated p53 (ac-p53), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), proteasome 20S core subunits, postsynaptic density protein 95 (PSD95), SIRT1, synaptophysin, acetylated tau (ac-tau), p-tau clone AT8, total tau clone HT7, and ubiquitin. Details of primary antibodies used are presented in Supplementary Table 1. Secondary antibodies were peroxidase-conjugated (1:2000) (GE Healthcare). Quantitative values of the correspondent bands were detected by a chemiluminiscence method using VersaDoc Imaging System 5000 (Bio-Rad, USA). Optical density of the studied proteins was normalized to actin or tubulin. Protein levels were calculated and expressed relative to the amount in the NoTg-Ct mouse group.

Proteasome activity assay

Proteasomal activity was evaluated in the brain cortex by the Proteasome-Glo™ Assay Systems (Promega, USA). Cortex tissues in ice-cold PBSE (PBS, 5 mM EDTA, pH 7.4) at a ratio of 1:10 (buffer/tissue; v/w) were sonicated on ice for 20 sec with a 1 sec pulse length, twice, using a pulsed homogenizer. Obtained tissue lysates were centrifuged at 13,000 *g* for 10 min at 4°C, and the supernatants were subjected to protein quantification employing the Bradford assay. The supernatants were diluted with cold PBSE at a concentration of 0.2 mg/ml total protein. A total of 10 µg of protein (50 µl of 0.2 mg/ml diluted extract) was added to 50 µl of the luminescent reagent containing the Ultra-Glo™ Luciferase and the specific luminogenic substrate (Suc-LLVY-Glo™ for the chymotrypsin-like activity assay, Z-LRR-Glo™ for the trypsin-like activity assay, or Z-nLPnLD-Glo™ for the caspase-like activity assay) in a 96-well plate. Solutions were mixed for 30 sec at 400 rpm and incubated for 30 min at room temperature. The resulting luminescence was measured twice with an integration time of 1 sec utilizing the Orion II Microplate Luminometer (Titertek-Berthold, Germany). In this setup, luminescence signal intensity corresponded to proteasomal proteolytic activity. The proteasomal inhibitor was used (MG-132, 10 µM) to calculate unspecific background activity.

Statistical analysis

Results are expressed as mean ± SEM. Data were analyzed with analysis of variance (ANOVA) procedures; factors were genotype and treatment. Two-way repeated measures ANOVA was employed to analyze the acquisition task of the MWM test. All other data were analyzed by regular two-way ANOVA followed by main effect analysis for comparison of groups where interaction between factors was present. Statistical analyses were performed using GraphPad Prism 6 and IBM SPSS Statistics v23.

Results

Resveratrol administration induced beneficial effects on BPSD-like behavior

Ten-month resveratrol treatment induced a significant protective effect against the AD-like pathology underlying BPSD-like behavioral alterations in 12 month-old mice (Fig. 1a-e). In the Open field test, 3xTg-Ct mice demonstrated lower vertical explorations (rearings) compared to NoTg mice (Fig. 1a). Resveratrol administration increased the number of total rearings in both NoTg-RV and 3xTg-RV mice [genotype, $F(1, 39) = 48.29$, $p < 0.0001$; and treatment, $F(1, 39) = 4.219$, $p = 0.0467$]. Moreover, 3xTg-Ct mice showed lower horizontal mobility compared to NoTg mice (Fig. 1b). Resveratrol treatment also increased the total distance covered in both strains [genotype, $F(1,42) = 40.75$, $p < 0.0001$; and treatment, $F(1,42) = 7.343$, $p = 0.0097$]. In the Boissier's 4 hole-board test, 3xTg-Ct mice showed higher latency for first-hole exploration compared to NoTg mice (Fig. 1c). Resveratrol treatment reduced latency in both NoTg-RV and 3xTg-RV mice [genotype, $F(1,42) = 28.88$, $p < 0.0001$; and treatment, $F(1,42) = 6.349$, $p = 0.0156$]. In the Dark & Light box test, 3xTg-Ct mice presented a higher anxiety response compared to NoTg mice (Fig. 1d-e). Resveratrol administration increased, in both mouse, strains the number of entries into the lit area (Fig. 1d) [genotype, $F(1,42) = 11.89$, $p = 0.0013$; and treatment, $F(1,42) = 5.027$, $p = 0.0303$] and the time spent in the lit area (Fig. 1e) [genotype, $F(1,42) = 9.360$, $p = 0.0039$; and treatment, $F(1,42) = 4.844$, $p = 0.0333$].

Resveratrol administration induced beneficial effects on cognitive behavior

Ten-month resveratrol treatment induced a significant protective effect against the AD-like pathology involved in learning and memory capacities (Fig. 2a-f). Cognition was preserved in 12-month-old 3xTg-RV mice, in addition to inducing cognitive enhancement effects in NoTg-RV mice. In the NOR test, 3xTg-Ct mice exhibited a deficit of recognition memory, while NoTg-RV and 3xTg-RV mice increased their capacity to remember familiar objects at 2 h (Fig. 2b) [genotype, $F(1,36) = 4.195$, $p = 0.0479$; and treatment, $F(1,36) = 8.826$, $p = 0.0053$] and at 24 h (Fig. 2c) [treatment, $F(1,36) = 6.759$, $p = 0.0134$; and interaction genotype × treatment, $F(1,36) = 4.256$, $p = 0.0464$]. In the MWM test, the distances covered to locate the platform decreased along the 6 days of place-task acquisition (Fig. 2d) in 3xTg-RV mice, similar to NoTg mice; however, two-way repeated measures ANOVA did not show significant differences between groups. Nevertheless, in learning retrieval, 3xTg-Ct mice swam at random in the pool unaware of the former position of the escape platform, while both NoTg groups and that of the 3xTg-RV mice remembered the quadrant where the platform was situated (Fig. 2e) [genotype, $F(1,42) = 5.537$, $p = 0.0234$; and interaction genotype × treatment, $F(1,42) = 6.645$, $p = 0.0135$], indicating better memory response after resveratrol treatment. In addition, resveratrol administration increased swimming speed in both strains (fig. 2f) [treatment, $F(1,42) = 4.081$, $p = 0.0498$].

Resveratrol administration induced neuroprotective effects against amyloid-β pathology

Analysis of immunoblotting from hippocampus tissue showed higher protein levels of total APP (Fig. 3a) in 3xTg-AD mice as compared with NoTg mice [genotype, $F(1,20) = 48.59$, $p < 0.0001$], as expected. Furthermore, the levels of Aβ peptides, such as APP-CTF (Fig. 3b) [genotype, $F(1,20) = 41.45$, $p < 0.0001$; treatment, $F(1,20) = 8.680$, $p = 0.0080$; and interaction genotype × treatment, $F(1,20) = 6.687$, $p = 0.0177$], Aβ 6e10 (Fig. 3c) [genotype, $F(1,15) = 10.45$, $p = 0.0056$; treatment, $F(1,15) = 6.976$, $p = 0.0185$; and interaction genotype × treatment, $F(1,15) = 4.709$, $p = 0.0465$], and sAPPβ (Fig. 3d) [genotype, $F(1,23) = 4.528$, $p = 0.0443$; and interaction genotype × treatment, $F(1,23) = 9.954$, $p = 0.0044$], were increased to a higher degree in 3xTg-Ct compared to NoTg mice, as characterized for AD pathogenesis. Resveratrol treatment induced a decrease in amyloid pathology, by a recovery of the APP-CTF (Fig. 3b), Aβ 6e10 (Fig.

3c), and sAPPβ (Fig. 3d) protein levels in 3xTg-RV mice, due to a decrease of BACE1 secretase levels (Fig. 3e) [treatment, $F(1,19) = 4.993$, $p = 0.0377$] and the increase of the neprilysin protease (Fig. 3f) [treatment, $F(1,20) = 5.334$, $p = 0.0317$] in both strains. These results confirm the effect of resveratrol on Aβ pathology mitigation. The attenuation of the amyloidogenic pathway and the increased proteostasis exerted an effect on both strains treated with resveratrol; however, no significant changes were observed in the levels of the neuroprotector sAPPα peptide (Supplementary Fig. 1a). Resveratrol increased secretase ADAM10 levels with borderline statistical significance (Supplementary Fig. 1b) [treatment, $F(1,16) = 4.218$, $p= 0.0567$]. Protease IDE was reduced in 3xTg-AD mice, but resveratrol did not change levels (Supplementary Fig. 1c) [genotype, $F(1,14) = 4.789$, $p = 0.0461$].

Resveratrol administration induced neuroprotective effects against tau pathology

Analysis of immunoblotting from hippocampal tissue revealed elevated protein levels of total tau (Fig. 3g) in 3xTg-AD mice as compared with NoTg mice [genotype, $F(1,20) = 24.36$, $p < 0.0001$], as expected. The protein levels of p-tau (Fig. 3h) [genotype, $F(1,24) = 10.72$, $p = 0.0032$; treatment, $F(1,24) = 10.11$, $p = 0.0040$; and interaction genotype × treatment, $F(1,24) = 5.313$, $p = 0.0301$], and of ac-tau (Fig. 3i) [genotype, $F(1,25) = 12.53$, $p = 0.0016$; treatment, $F(1,25) = 8.924$, $p = 0.0062$; and interaction genotype × treatment, $F(1,25) = 5.562$, $p = 0.0265$] were increased to a greater degree in 3xTg-Ct compared to NoTg mice, as characterized for AD pathogenesis. Resveratrol treatment protected against tau pathology, in that it normalized p-tau (Fig. 3h) protein levels in 3xTg-RV mice, due to a decrease of ac-tau (Fig. 3i) protein levels in 3xTg-RV mice. Deacetylation of tau protein allows it to be degraded by the UPS. These results confirm the effect of resveratrol on tau pathology mitigation.

Resveratrol administration enhanced ubiquitin-proteasome system activity

Immunoblotting analysis demonstrated higher Hsp70 protein levels (Fig. 4a) [genotype, $F(1,20) = 35.84$, $p < 0.0001$; treatment, $F(1,20) = 6.283$, $p = 0.0209$; and interaction genotype × treatment, $F(1,20) = 7.517$, $p = 0.0126$] and ubiquitinated proteins levels (Fig. 4b) [genotype, $F(1,18) = 5.867$, $p = 0.0262$; treatment, $F(1,18) = 10.53$, $p = 0.0045$; and interaction genotype × treatment, $F(1,18) = 6.450$, $p = 0.0205$] in 3xTg-Ct compared to the hippocampus of NoTg mice. Resveratrol treatment restored Hsp70 (Fig. 4a) and ubiquitinated (Fig. 4b) protein levels in 3xTg-RV mice. Moreover, resveratrol treatment induced an enhancement of proteasome 20S core subunits levels (Fig. 4c) [treatment, $F(1,28) = 12.34$, $p = 0.0015$] in the hippocampus of NoTg-RV and 3xTg-RV mice. A tendency to a decrease in proteasome protein levels in 3xTg-Ct mice did not reach significance. Besides, resveratrol also induced enhancement of proteasome 20S core subunits levels (Fig. 4d) [treatment, $F(1,20) = 11.02$, $p = 0.0034$] in the cerebral cortex of both strains. Accordingly, resveratrol treatment induced an increase of trypsin-like activity (Fig. 4e) [treatment, $F(1,29) = 7.638$, $p = 0.0098$] in the cerebral cortex of both strains, but no changes were detected in chymotrypsin-like (Supplementary Fig. 2a) and caspase-like activity (Supplementary Fig. 2b). These results showed the neuroprotective effects of resveratrol for aberrant proteins disposal by enhancement of the brain proteasome function.

Resveratrol administration activates SIRT1 pathway regulators

Immunoblotting analysis did not show significant variations of SIRT1 protein levels in the hippocampus of both strains, or after resveratrol treatment (Fig. 5a). However, SIRT1 activity was confirmed by the diminution of p53 acetylated in both strains after resveratrol treatment, indicative of SIRT1 deacetylation action (Fig. 5b) [treatment, $F(1,20) = 9.208$, $p = 0.0065$]. Moreover, resveratrol treatment incremented p-AMPK protein levels (Fig. 5c) [treatment, $F(1,23) = 8.867$, p

= 0.0067] in both strains, which subsequently produces an increase of the substrate NAD^+, indicative of SIRT1 pathway activation. Resveratrol promoted the increase of p-CREB (Fig. 5d) [treatment, $F(1,20) = 15.75$, $p = 0.0008$] by SIRT1 pathway in both strains. Moreover, PGC-1α protein levels were lower in 3xTg-AD compared to NoTg mice, indicative of mitochondria dysfunction (Fig. 5e); however, resveratrol administration increased protein levels in both strains [genotype, $F(1,23) = 8.937$, $p = 0.0065$; treatment, $F(1,23) = 7.419$, $p = 0.0121$].

Resveratrol administration does not modulate neurotrophism or plasticity.

Immunoblotting demonstrated that PSD95 (Supplementary Fig. 3a) [genotype, $F(1,16) = 21.79$, $p = 0.0003$], and Synaptophysin (Supplementary Fig. 3b) [genotype, $F(1,23) = 5.960$, $p = 0.0227$] protein levels were higher in NoTg as compared with 3xTg-AD hippocampal tissue. However, resveratrol treatment had no effect, and protein levels were unchanged.

Discussion

Chronic administration of resveratrol in the 3xTg-AD mouse model of AD, and in normal NoTg mice, confirmed its potential usefulness for the treatment and prevention of AD, and further extended previous mechanisms in findings from *in vitro* [38,57,58] and *in vivo* studies [20,22,23,26,59,60].

Our results showed that resveratrol administration induced total protection against cognitive loss in 3xTg-AD mice and memory enhancement in control mice, in hippocampus-based tests of learning and memory. The hippocampus is an area selectively affected by AD [61], and the deterioration of hippocampal circuits contributes greatly to the devastating effects of memory loss in the disease [62]. Several regions of cerebral cortex are also deeply affected by AD pathology [63]. Both hippocampus and cerebral cortex shown accumulation of Aβ and p-tau and neurodegenerative changes in 12 months old 3xTg-AD mice [53].

The spatial learning and memory analyzed in the MWM test are considered to be associated with optimal functioning of hippocampal circuits [64,65]. Untreated 3xTg-AD mice exhibited deficient learning and impaired retention in the MWM task, as reported previously [52]. This task, which is dependent on the dorsal hippocampus [66], revealed totally protection in 3xTg-AD mice by means of resveratrol administration. Furthermore, 3xTg-AD mice showed impairment of recognition memory evaluated by the NOR test [67], a task involving the hippocampus and brain cortex regions [68,69]. Recognition memory was also preserved by resveratrol administration in 3xTg-AD mice. The neuroprotection of resveratrol against cognitive impairment in 3xTg-AD mice confirmed previous studies in the SAMP8 mouse model of pathological aging and AD [26,59] and in APP/PS1 AD transgenic mice [22]. Furthermore, recognition memory was generally improved by resveratrol, demonstrating cognitive enhancement in NoTg mice. Benefits of resveratrol administration were also proven by reversal of the abnormal behaviors included in the BPSD phenotype, which comprise very prevalent neuropsychiatric symptoms in patients with AD [70]. In these non-cognitive behaviors, resveratrol also exhibited beneficial effects in NoTg mice, which is indicative of enhanced well-being, such as increased exploration and decreased anxiety behaviors. Considering the results of cognitive and non-cognitive behavior, a preventive and therapeutic effect of resveratrol against AD dementia has been demonstrated. The benefits in neuronal activity demonstrated in control-strain mice suggest an enhancement in brain resilience that would decrease the risk of AD.

Analysis of brain pathological changes in 3xTg-AD mice demonstrated that resveratrol induced a decrease in amyloid and tau pathologies to levels similar to those in the control strain. Only higher levels of APP and total tau were observed in all 3xTg-AD mouse groups compared to NoTg mice, in agreement to their transgene expression [51]. Western blot immunodetection results of amyloidogenic fragments (Aβ and CTF) were conclusive of total protection. The fight against the cerebral excess of Aβ is one of the main objectives of therapies in clinical studies [71]. The origin of the excess of Aβ in the brain is not known, although both increased generation and unbalanced degradation are assumed [72]. The non-amyloidogenic pathway appears to be neuroprotective, while the amyloidogenic pathway generates neurotoxic Aβ peptides [73]. Both pathways compete with each other, since increasing α-secretase activity reduces production of the Aβ peptides [74,75]. BACE1 is regarded as a key target for therapeutic interventions in AD because it is one of the main responsible for Aβ generation in the brain [76,77]. Targeted deletion of BACE1 in APP transgenic mice completely abolishes the production and deposition of Aβ and also rescues memory deficits [78]. We found a reduction of the amyloidogenic secretase BACE1 by resveratrol in both 3xTg-AD and NoTg strains, thus indicating a shift to the non-amyloidogenic pathway of APP processing. Peptide sAPPβ was higher only in 3xTg-AD and resveratrol reduced the protein levels. One of the most important amyloid degrading enzymes is neprilysin, which plays a major role in degrading Aβ. Administration of resveratrol promoted the increase in neprilysin protein levels, contributing to

the anti-amyloidogenic effect of resveratrol in both strains. Gene or cell therapy mediated increase of neprilysin is sufficient to ameliorate AD-like phenotypes in several mouse models [79-81]. Our results suggest that resveratrol reduced Aβ load through the decrease of amyloidogenic secretase BACE1 and by means of the increase of amyloid-degrading enzyme neprilysin levels. Supplementation of resveratrol also induced a trend toward increasing the levels of ADAM10 in both strains, altogether contributing to neuroprotection and cerebral resilience. SIRT1 decreases Aβ production [30,82,83]; therefore, activation of SIRT1 might at least partially mediate the anti-amyloid pathological effects of resveratrol. Resveratrol revealed outstanding protection against tau pathology in 3xTg-AD mice. Tau pathology is proposed to be triggered by amyloid pathology in the AD brain [84]. However, 3xTg-AD neurons, in addition to the APP and PS1 familial AD genes, express a human tauopathy gene, thus stressing tau pathology in this mouse model. Tau is one of the therapeutic targets in AD [85]. We found that the increase of p-tau levels in 3xTg-AD mice was paralleled by an increase in tau acetylation. Acetylation of lysine residues has been reported as a novel modification in the brain tissue of patients with AD and familial tauopathies [86-88]. Resveratrol administration reduced p-tau levels in 3xTg-AD mice, which may occur through the deacetylation of the tau protein by SIRT1, thereby favoring degradation of p-tau by the proteasome pathway. It is known that activation of SIRT1 pathway has a positive effect on the reduction of p-tau formation [86] and mice with a SIRT1 deletion show an accumulation of ac-tau in the brain [86,88].

The enhancement of proteolysis systems shown here by resveratrol may be chief in both prevention and therapy against AD and in neurodegenerative diseases coursing with the accumulation of aberrant proteins. We found a normalization of Hsp70 and ubiquitin levels in 3xTg-AD and a significant increase of proteasome levels and enzymatic activity in both NoTg and 3xTg-AD mice. UPS is the major proteolytic system that degrades aberrant proteins, including Aβ and p-tau [50]. Loss of proteasome activity increases the risk of AD, representing a clear link between this neurodegenerative disease and the aging process [40]. Functional proteasome degrades ubiquitin-tagged misfolded or aggregated proteins. Our results are in agreement with the previous observation that resveratrol promotes the intracellular degradation of Aβ in cell lines by a mechanism that implicates the proteasome [38]. SIRT1 is known to be involved in the maintenance of quality control of proteins mediated by UPS *in vitro* [30,89]; however, an effect of resveratrol on UPS activation had not been reported previously *in vivo*. The chaperone Hsp70 is involved in the degradation of aberrant proteins through interaction with CHIP and the ubiquitin E3 ligase [48,90,91]. Resveratrol induced a further decrease of Hsp70, in agreement with SIRT1 regulation [48], and also normalized ubiquitinated protein levels in 3xTg-AD mice, suggesting a recovery of UPS functionality. Proteasome 20S core subunits levels were decreased in 3xTg-AD mice, indicating impairment of the proteasome function, in agreement with previous results in AD brain tissue [92] and in hippocampal homogenates of 3xTg-AD mice [93]. Resveratrol enhanced the levels of proteasome 20S core subunits in both hippocampus and cortex tissue of NoTg and 3xTg-AD mice, and trypsin proteasomal activity in cerebral cortex of both strains of mice, suggesting an enhancement of UPS functionality. Some neurofibrillary tangles of p-tau are ubiquitinated [94,95], and neuronal death appears to be the end-point for neurofibrillary degeneration [96]. The increased yield of proteasome protein levels in brain tissue of 3xTg-AD mice would lead to the total degradation of aberrant Aβ and p-tau proteins, so that ubiquitinated proteins and Hsp70 were restored to baseline levels. Resveratrol also induced proteostasis enhancement in NoTg mice; thus, this is, to our knowledge, the first time reported that resveratrol increases proteasome function and ameliorates AD-like pathology *in vivo*. We highlight the increase of both the proteasome and neprilysin in the strain of NoTg mice, which would induce resilience against the accumulation of abnormal proteins.

Although resveratrol was initially shown to directly activate SIRT1 in an assay utilizing a fluorophore-linked substrate [97], recent studies have shown that resveratrol indirectly activates SIRT1 due to its effect on cAMP signaling [34].

SIRT1 is a nuclear localization protein [98] that, catalyzes the deacetylation of histones and several transcription factors through the consumption of the substrate NAD^+ [29,99]. Resveratrol is thought to elicit its beneficial effects through upregulation of the AMPK/SIRT1 pathway [100-102]. It is suggested that resveratrol enhances AMPK activity, which in turn increases NAD^+ concentration, resulting in the activation of SIRT1 [34,35,103]. Accordingly, AMPK-deficient mice showed to be resistant to the metabolic effects of resveratrol [101]. We found higher levels of p-AMPK in the hippocampus of both resveratrol-treated groups of mice; however, we did not observe changes in SIRT1 protein levels. In the inducible p25 transgenic mouse model of AD and tauopathies, introduction of resveratrol directly into the brain ventricles prevented learning impairment, reduced hippocampal neurodegeneration, and decreased acetylation of the SIRT1 substrate p53 [23]. SIRT1 induces neuroprotective effects against AD pathology through regulating the acetylation homeostasis of key proteins [29]. Accordingly, a decrease in p53 acetylation indicates SIRT1 activation in mouse hippocampus.

The cyclic-AMP responsive element binding protein (CREB) is a basic leucine zipper transcription factor and a downstream target of ERK signaling during hippocampal-dependent learning [104]. The transcription of several downstream neuroprotective molecules is regulated by p-CREB. Deficiencies in CREB signaling have been linked to neurodegenerative processes and AD [105]. In previous studies, elevated p-CREB levels were found in the hippocampal CA1 region of resveratrol-treated rats [106]. Furthermore, it has been demonstrated that resveratrol can modulate learning and memory function by modulating SIRT1 and regulating p-CREB expression [60]. SIRT1 can regulate mitochondrial biogenesis, contributing to the maintenance of functional mitochondria [107]. It is also well-established that SIRT1 regulates the activity and acetylation status of PGC-1α [103,108,109], and many studies have pointed out the ability of resveratrol to upregulate PGC-1α activity[110], which results in beneficial changes in the mitochondrial function [100,111,112]. Previous studies indicate the deficiencies of mitochondrial complexes in 3xTg-AD mice [54] and elevated levels of oxidative lesions and alterations of antioxidant enzymes [52,113]. In this regard, we cannot discard some contribution of direct antioxidant mechanisms of reveratrol or other protective effects of this pleiotropic molecule [14,114]. Mitochondrial dysfunction is a molecular marker of aging that establishes a connection between aging and the risk of AD [115,116]. Mitochondrial dysfunction can be ameliorated by inducing PGC-1α via resveratrol-mediated modulation of AMPK [117,118]. The enhancement of AMPK [35], PGC-1α [119] and CREB [60] pathways in all the mice treated with resveratrol corroborates the beneficial changes in mitochondrial function and plasticity processes, which will induce effector ways of protecting mitochondria, thus increasing the resilience of the brain.

Conclusions

In summary, diet supplementation with resveratrol led to complete protection against memory loss in 3xTg-AD mice and to cognitive enhancement in healthy NoTg mice. Furthermore, resveratrol improved non-cognitive behaviors indicative of well-being in both mouse strains. Analysis of resveratrol administration in AD and healthy mice led to the uncovering of the following novel resveratrol mechanisms *in vivo*: i) activation of neprilysin and downregulation of BACE1, which reduces amyloid load; ii) enhancement of UPS, which leads to a reduction of aberrant amyloid and tau proteins, and iii) upregulation of AMPK/SIRT1 pathways, leading to an increase of PGC-1α and CREB. A schematic representation of the proposed mechanisms activated by resveratrol in this study is depicted in Fig. 6. The results depicted here suggest resveratrol-induced activation of SIRT1 as the main pathway inducing potent neuroprotective effects. This natural polyphenol has a potential in AD prevention by increasing brain resilience against aberrant proteins.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Figure Legends

Fig. 1 Resveratrol treatment induced protection against BPSD-like behavior. Total number of rearings (**a**) and distance covered (**b**) in the Open field test. Latency of first-hole exploration (**c**) in the Boissier's 4 hole-board test. Number of entries in the lit area (**d**), and time spent in the lit area (**e**) in the Dark & Light box test. Values are mean ± SEM (n = 10-14). Statistical analysis: Two-way ANOVA, effect of genotype &&p < 0.01 and &&&p < 0.001; and effect of treatment $p < 0.05 and $$p < 0.01

Fig. 2 Resveratrol administration induced protection against cognitive loss. NOR test at times 0h (**a**), 2h (**b**), and 24h (**c**). MWM test with distances covered to reach platform (**d**), distance covered in platform quadrant after removal (**e**), and swimming speed (**f**). Values are mean ± SEM (n = 8-14). Statistical analysis: **c**, **e** Two-way ANOVA, *p < 0.05 and ***p < 0.001 compared to NoTg mice; #p < 0.05 and ##p < 0.01 compared to control treatment; **d** Two-way repeated measures ANOVA; **b**, **f** Two-way ANOVA, effect of genotype &p < 0.05; and effect of treatment $p < 0.05 and $$p < 0.01

Fig. 3 Resveratrol treatment protects against Aβ and tau pathology in hippocampus. Western blot analysis of total APP (**a**), APP-CTF (**b**), Aβ fragment (**c**), sAPPβ (**d**), BACE1 (**e**), neprilysin (**f**), total tau (**g**), p-tau (**h**) and ac-tau (**i**) in the hippocampus of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 4-8). Statistical analysis: **a, d, f, g** Two-way ANOVA, effect of genotype &&&p < 0.001; and effect of treatment $p < 0.05; **b, c, e, h, i** Two-way ANOVA, **p < 0.01 and ***p < 0.001 compared to NoTg mice; #p < 0.05 and ##p < 0.01 compared to control treatment

Fig. 4 Resveratrol administration enhances the activity of the ubiquitin-proteasome system. Protein analysis of Hsp70 (**a**), ubiquitinated proteins (**b**) and proteasome 20S core subunits (**c**) in the hippocampus of 3xTg-AD and NoTg mice. Protein analysis of proteasome 20S core subunits (**d**) and proteasome trypsin-like activity (**e**) in the cerebral cortex tissue of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 5-11). Statistical analysis: **a, b** Two-way ANOVA, **p < 0.01 and ***p < 0.001 compared to NoTg mice; ##p < 0.01 compared to control treatment; **c, d, e** Two-way ANOVA, effect of treatment $$p < 0.01

Fig. 5 Resveratrol administration activates SIRT1 pathway by activation of p-AMPK. Protein analysis of SIRT1 (**a**), ratio of p53 acetylated to total p53 (**b**), ratio of p-AMPK to total AMPK (**c**), ratio of p-CREB to total CREB (**d**), and PGC-1α (**e**) in the hippocampus of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 5-7). Statistical analysis: Two-way ANOVA, effect of genotype &&p < 0.01; and effect of treatment $p < 0.05, $$p < 0.01 and $$$p < 0.001

Fig. 6 Proposed pathways involved in the neuroprotective effects of resveratrol administration, leading to a reduction in AD-like pathology through proteostasis enhancement. See text for discussion of mechanisms. Abbreviations: Ac, acetylated; Aβ, amyloid-β; AD, Alzheimer's disease; ADAM10, a disintegrin and metalloproteinase 10; AMPK, adenosine monophosphate-activated protein kinase; BACE1, beta-site APP cleaving enzyme 1; CREB, cAMP response element-binding protein; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; p-tau, hyperphosphorylated tau; UPS, ubiquitin proteasome system.

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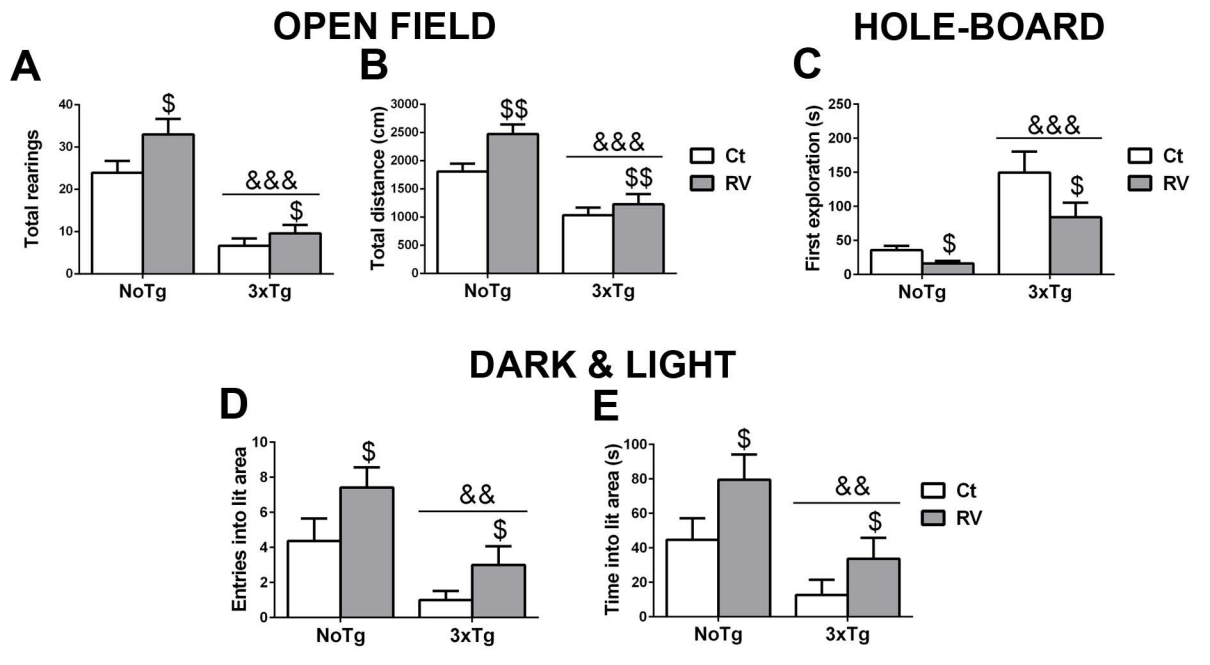
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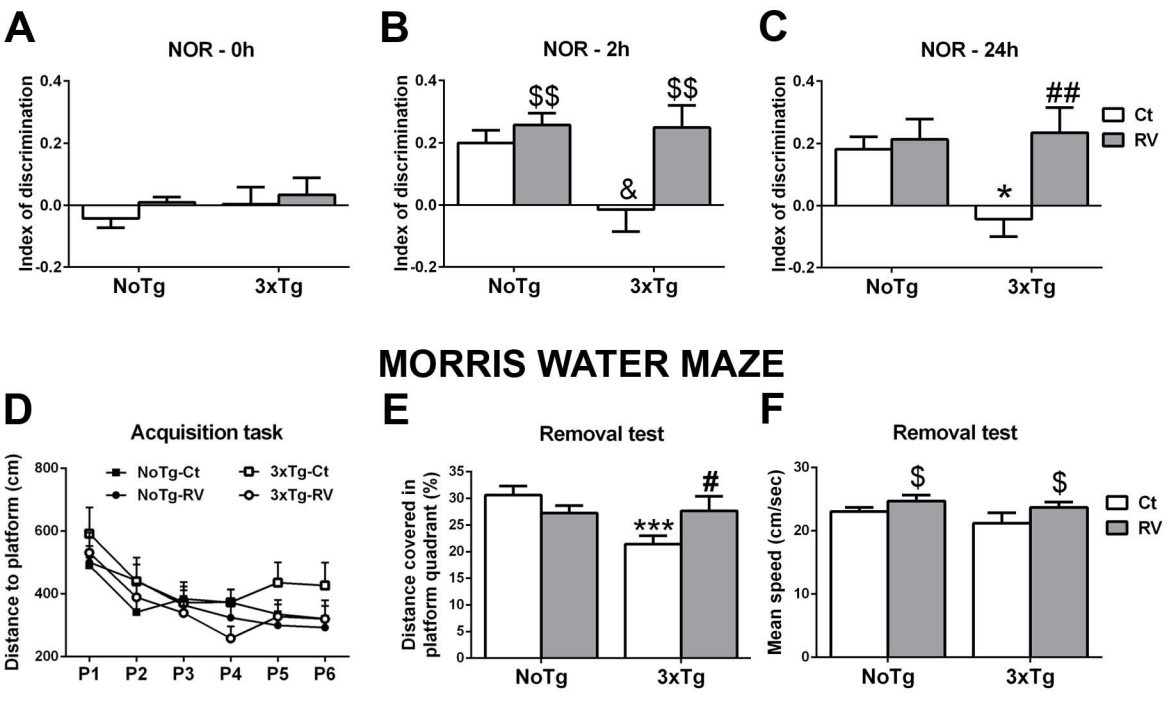
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MORRIS WATER MAZE
D
Acquisition task
Distance to platform (cm)
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400
200
NoTg-Ct
NoTg-RV
3xTg-Ct
3xTg-RV
P1
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P6
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Removal test
Distance covered in platform quadrant (%)
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Removal test
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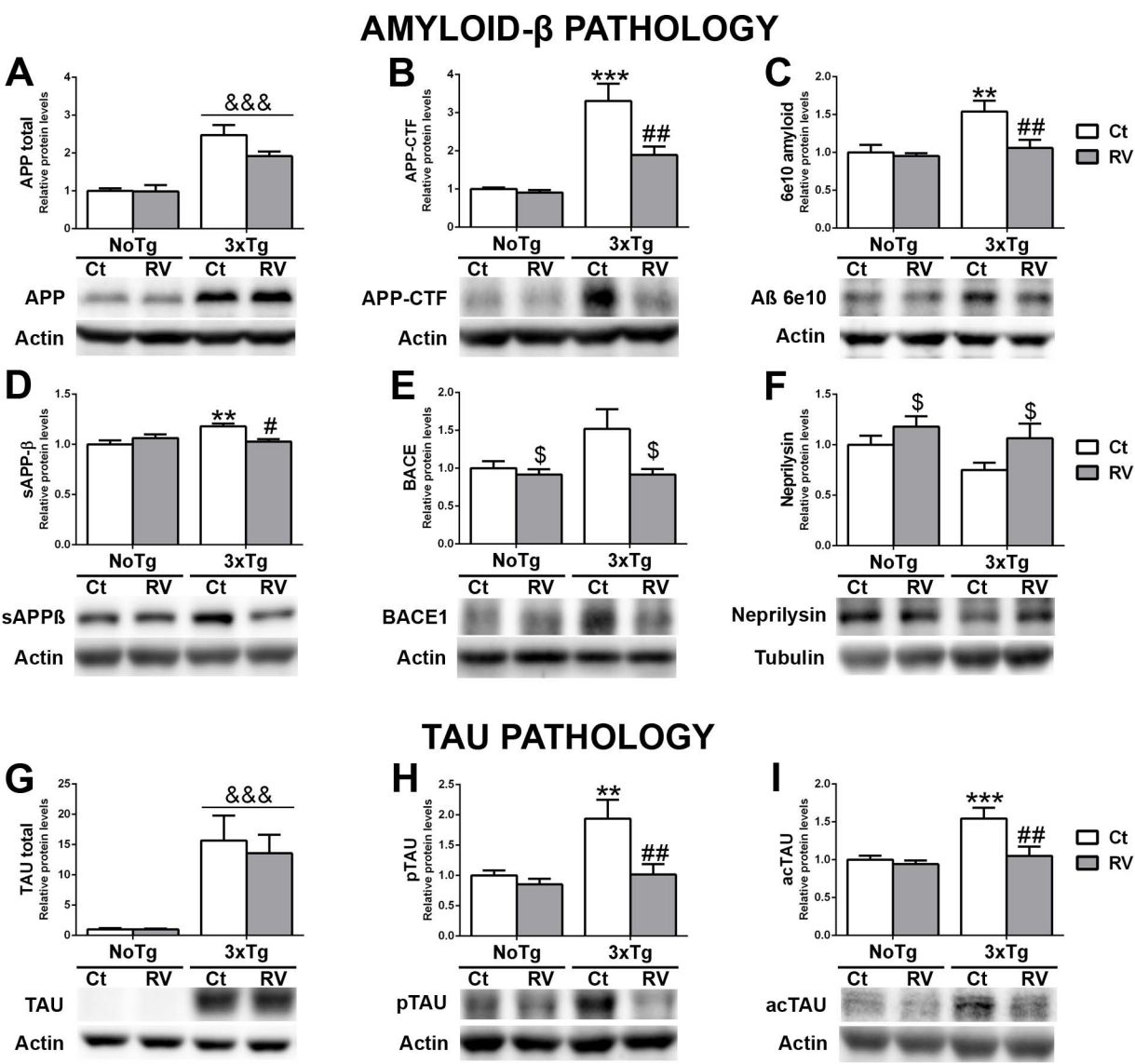
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NoTg
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E
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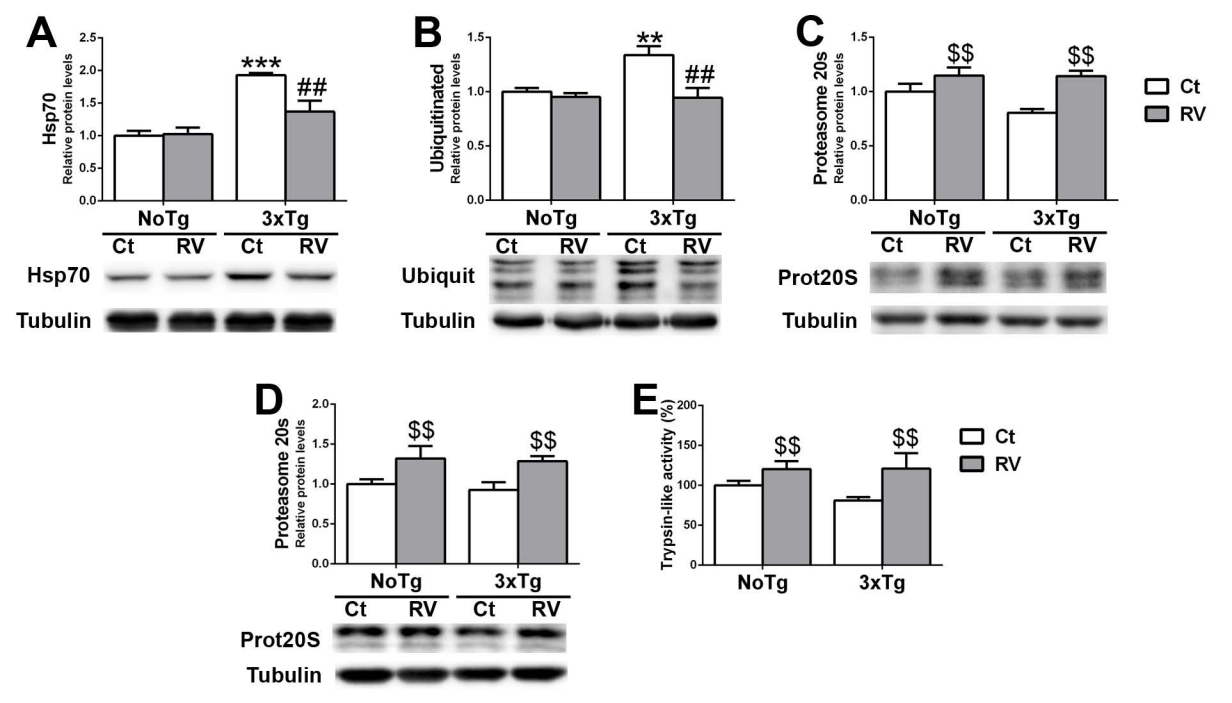
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NoTg
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Ct
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Hsp70
Tubulin
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Ubiquitinated
Relative protein levels
1.5
1.0
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##
NoTg
3xTg
Ct
RV
Ct
RV
Ubiquit
Tubulin
C
Proteasome 20s
Relative protein levels
1.5
1.0
0.5
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NoTg
3xTg
Ct
RV
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RV
Prot20S
Tubulin
Ct
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D
Proteasome 20s
Relative protein levels
2.0
1.5
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NoTg
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Trypsin-like activity (%)
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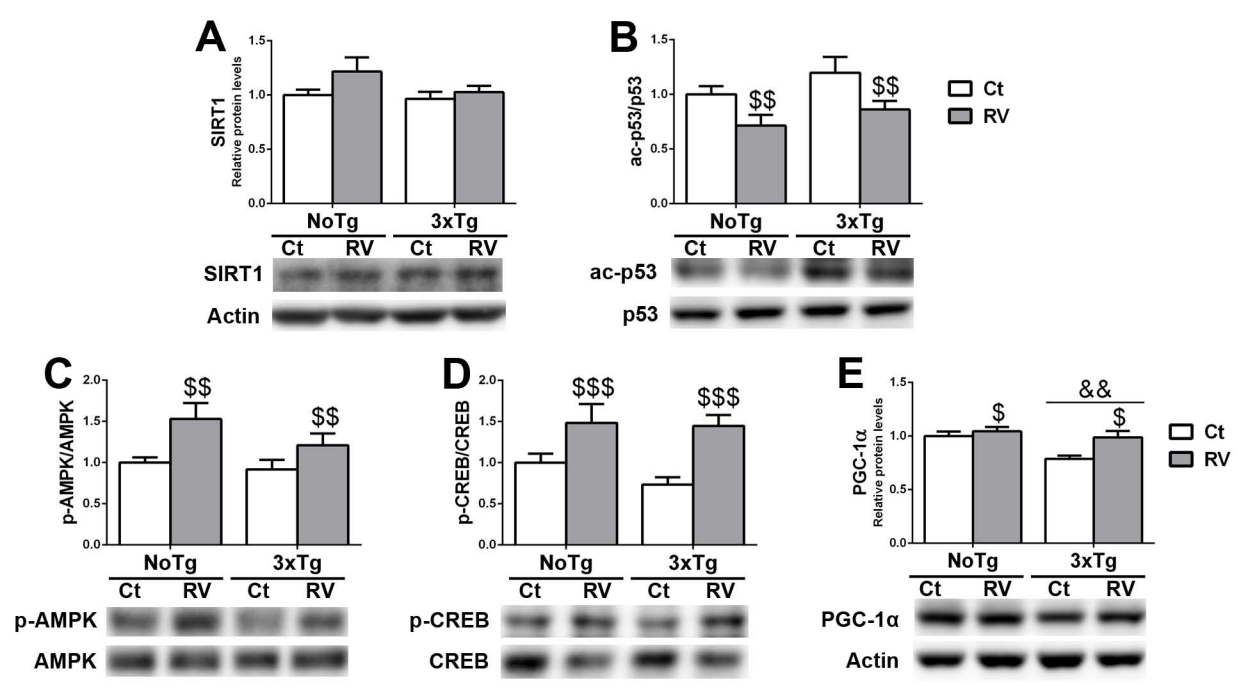
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Relative protein levels
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1.0
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NoTg
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Actin
B
ac-p53/p53
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RV
ac-p53
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C
p-AMPK/AMPK
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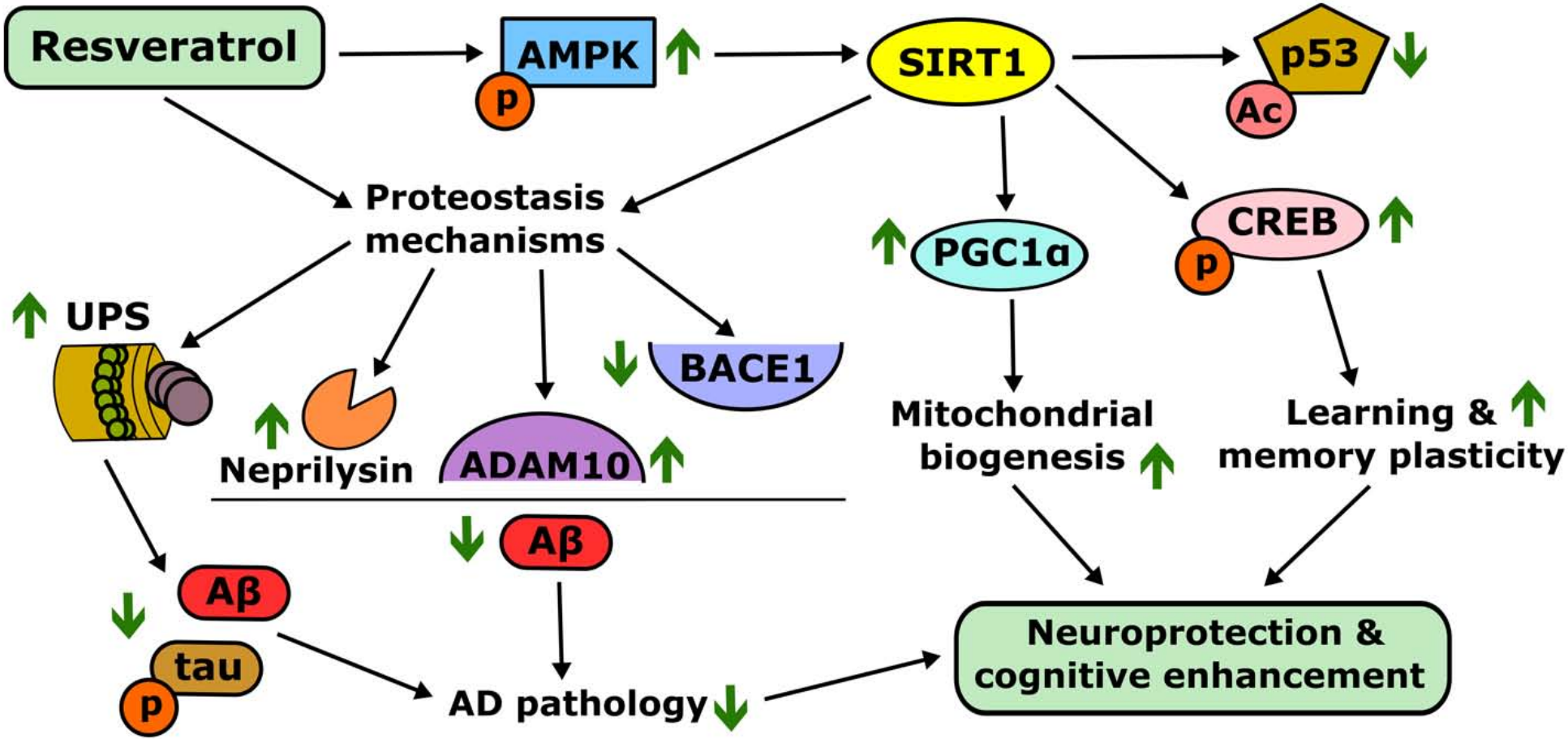
Resveratrol
AMPK
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SIRT1
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Proteostasis
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PGC1α
CREB
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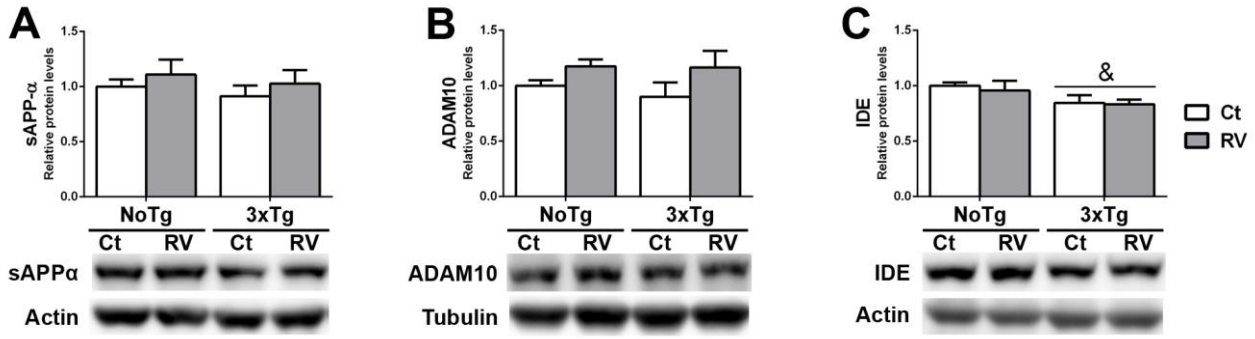
UPS
BACE1
Neprilysin
ADAM10
Mitochondrial
biogenesis
Learning &
memory plasticity
Aβ
Aβ
tau
p
AD pathology
Neuroprotection &
cognitive enhancement

Supplementary Table 1

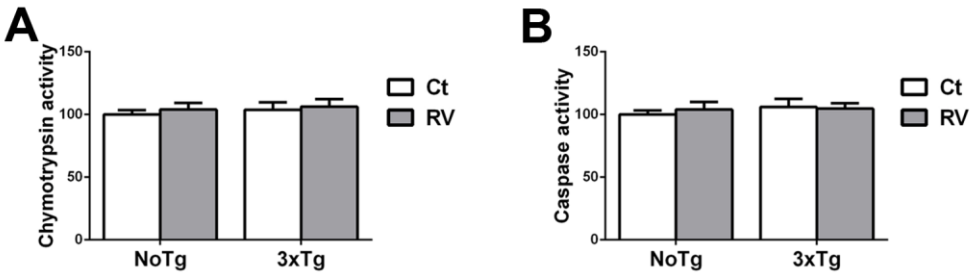
List of primary antibodies used in Western blotting (WB)

Antibody	Dilution	Source	Catalog Nº
Aβ, clone 6e10	1:1000	BioLegend	803001
sAPPα	1:500	BioLegend	813501
sAPPβ	1:1000	BioLegend	813401
APP-CTF, clone C1/6.1	1:1000	Covance	SIG-39152
Actin (20-33)	1:10 000	Sigma	A5060
ADAM10	1:1000	Abcam	ab1997
AMPK	1:1000	Cell Signaling	2532S
p-AMPK (Thr172)	1:1000	Cell Signaling	2535S
BACE1	1:1000	Abcam	ab2077
CREB	1:1000	Cell Signaling	9197S
p-CREB (Ser133)	1:1000	Cell Signaling	9196S
Hsp70 (W27)	1:2000	Calbiochem	HSP01
IDE	1:1000	Calbiochem	PC730
Neprilysin/CD10	1:1000	R&D system	AF1126
ac-p53, acetyl K382	1:1000	Abcam	ab37318
PGC-1α	1:500	Santa Cruz Biotechnology	sc-13067
Proteasome 20S core	1:1000	Enzo Life Sciences	BML-PW8155
PSD95	1:500	Millipore	MAB1598
SIRT1	1:2500	Cell Signaling	2028s
Synaptophysin	1:10 000	Dako	A0010
ac-tau, acetyl K280	1:500	AnaSpec	56077
p-tau, clone AT8	1:1000	Thermo Scientific	MN1020
Total tau, clone HT7	1:1000	Thermo Scientific	MN1000
β-tubulin	1:10000	Sigma	T4026
Ubiquitin	1:2000	Abcam	ab137031

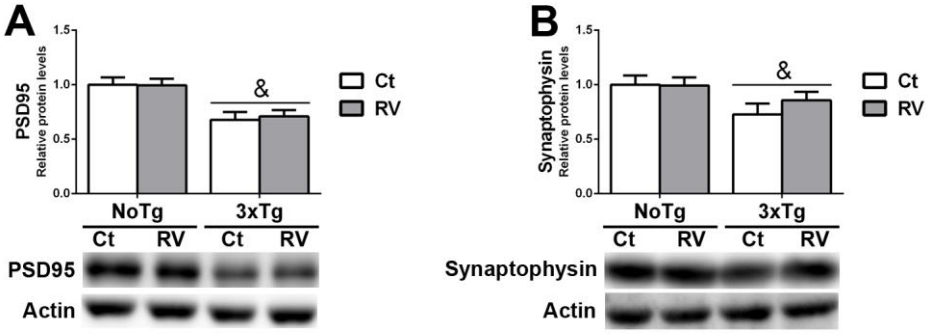
Supplementary figures



Supplementary Fig. 1 Resveratrol administration has minor effect on the non-amyloidogenic pathway and IDE. Protein analysis of sAPPα peptide (**a**), ADAM10 (**b**) and IDE (**c**) in the hippocampus of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 4-7). Statistical analysis: Two-way ANOVA, effect of genotype &p < 0.05.



Supplementary Fig. 2 Resveratrol administration does not modulate chymotrypsin-like and caspase-like activity. Chymotrypsin-like activity (**a**) and Caspase-like activity (**b**) in the cortex tissue of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 5-11).



Supplementary Fig. 3 Resveratrol does not modulate neurotrophism or plasticity. Relative protein levels for PSD95 (**a**) and Synaptophysin (**b**) in the hippocampus of 3xTg-AD and NoTg mice. Values are mean ± SEM (n = 5-7). Statistical analysis: Two-way ANOVA, effect of genotype &p < 0.05.