Published in final edited form as:

Food Funct. 2020 December 01; 11(12): 10351–10361. doi:10.1039/d0fo02438d.

Effects of (−)-Epicatechin on Neuroinflammation and Hyperphosphorylation of Tau in Hippocampus of Aged Mice

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Abstract

Evidence has implicated oxidative stress (OS) and inflammation as drivers of neurodegenerative pathologies. We previously reported on the beneficial effects of (−)-epicatechin (Epi) treatment, on aging-induced OS and its capacity to restore modulators of mitochondrial biogenesis in the prefrontal cortex of 26-month-old male mice. In the present study using the same mouse model of aging, we examined the capacity of Epi to mitigate hippocampus OS, inflammation, hyperphosphorylation of tau protein, soluble β-amyloid protein levels, cell survival, memory, anxiety-like behavior levels as well as systemic inflammation. Mice underwent 4 weeks of Epi treatment (1 mg/kg/day) and samples of hippocampus were obtained. Assessments of the OS markers protein carbonyls and malondialdehyde levels demonstrated significant increases (~3 fold) with aging that were partially suppressed by Epi. Protein levels of the glial fibrillary acidic protein, inflammatory factor 1 (Iba1), proinflammatory cytokines, interleukins (IL-1β, IL-3, 5, 6 and 15), ciclooxygenase 2, tumor necrosis factor α, nuclear factor activated B cells and interferon γ increase with aging and were also significantly decreased with Epi treatment. However, anti-inflammatory cytokines, IL-1ra, Il-10 and 11 were decreased in aging and were restored with Epi. Epi also reversed aging effects on the hyperphosporylation of tau, increased soluble β-amyloid levels (~2 fold), cell death, (as per caspase 3 and 9 activity), and reductions in nerve growth factor and triggering receptor myeloid cells 2 levels. Measures of anxiety like behavior and memory demonstrated improvements with Epi treatment. Indicators of systemic inflammation were elevated with aging and Epi was capable to decrease blood inflammatory markers. Altogether,

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Conflicts of Interest

Dr. Villarreal (co-founder) and Dr. Ceballos are sotckholders of Epirium Inc

results evidence a significant capacity of Epi to mitigate hippocampus OS and inflammation leading to improved brain function.

Keywords

Aging; oxidative stress; memory; anxiety

Introduction

Aging is associated with cognitive decline which has been linked to a progressive loss of specific neuronal cell populations and to the presence of protein aggregates in the brain[1]. In the setting of aging, common features include high levels of local (i.e. brain) and systemic oxidative stress (OS) as well as inflammation which can promote neurodegeneration and/or neuronal cell death[1–3]. In the aged brain, extracellular amyloid plaques (senile plaques) and intracellular neurofibrillary tangles (NFTs) develop secondary to the β-A aggregates and hyperphosphorylation (hp) of the microtubule-binding protein tau respectively[4]. Critical to the preservation of neuronal health is the presence and activity of nerve growth factor (NGF) and triggering receptor expressed on myeloid cells2 (TREM2) whose levels also fall with aging[5,6]. Conversely, with aging and when there is trauma or disease, glial fibrillary acidic protein (GFAP) expression increases, therefore it can serve as a biomarker of neurotoxicity[7,8]. To date there are not effective therapies aimed at the preservation of neuronal health and thus, the identification of safe and effective agents that exert these actions is of high priority.

Preclinical, clinical and epidemiological evidence indicates that the consumption of cacao products or specific flavanols found in cacao, can ameliorate age-related cognitive decline. A study using aged rats demonstrated positive effects of a flavonoid-rich cocoa extract on performance in discrimination and spatial tasks[9]. In normal aged human subjects, the consumption of flavanol rich cocoa for 8 weeks was associated to improvements in cognitive function, linked to enhanced hippocampal function and/or brain blood flow[10–16]. Epidemiological studies also report on the association between the sustained consumption of cocoa products (most prominently dark chocolate) and improved cognitive function[17].

The most abundant flavanol present in cocoa is (−)-epicatechin (Epi), which stimulates nitric oxide production in endothelial cells and thus, increases blood flow. Epi can cross the blood-brain barrier and potentially act directly on neurons and supporting systems[18]. In mice, Epi favorably impacts anxiety[19], learning and memory[20,21] and such effects are associated with increased angiogenesis and neuronal spine density as well as with the upregulation of mRNA levels of proteins associated with learning and downregulation of markers of neurodegeneration in the hippocampus. We reported on the beneficial effects of Epi on OS levels and indicators of mitochondrial biogenesis, structure and function in the prefrontal cortex of aged mice (26-month-old)[22]. Thus, Epi recapitulates the beneficial actions of cocoa in blood vessels and brain. Nevertheless, no studies have examined the effects of Epi treatment on indicators of OS, neuroinflammation and neurodegeneration in the senile hippocampus and on systemic indicators of inflammation.

With these goals in mind, a study was implemented in young and aged mice assessing the effects of Epi treatment on blood cytokines levels, hippocampus OS markers, neuroinflammation modulators, hp-tau, soluble β-amyloid levels (and related signaling pathways) as well as markers of neuronal health and cell survival. Behavioral studies evaluated Epi effects on cognitive function and anxiety like behavior measures.

Materials and Methods

Animal studies

C57BL6 male mice were purchased from Jackson laboratories and divided into four groups. Animals were housed under controlled conditions, with 12 h light/dark cycles. Group 1, young control (YC) mice (6-month old), $n = 7$; group 2, aged control (AC) mice (26-month old), $n = 7$; group 3, Y mice treated with Epi (YE), $n = 7$; group 4, aged (A) mice treated with Epi (AE), $n = 7$. Groups 1 and 2 were provided vehicle (water) by oral gavage. As previously described[23] groups 3 and 4 were treated with Epi (1 mg/kg/day) by oral gavage during 4 weeks. Open field and object recognition tasks were performed in all four groups of mice. Results compared the effects of vehicle vs. Epi treatment measured at 4 weeks of treatment. At the end of treatment, blood samples and brain were collected, and the hippocampal region was dissected and stored at −80°C until used. All animal handling procedures were approved by the UCSD's Institutional Animal Care and Use Committee (approval number S10223) and according to NIH guidelines on the use of animals.

Protein Carbonylation

Protein carbonylation was used as a surrogate indicator of tissue OS levels[25]. Tissue samples were homogenized with a polytron tissue homogenizer (Brinkmann Instruments, Westbury, New York) in 500μl of cold buffer (50 mM 4-morpholineethanesulfonic acid, pH 6.7, containing 1 mM EDTA) and centrifuged (10,000 g) for 15 min at 4 °C. Supernatants were collected and incubated at room temperature for 15 min with streptomycin sulfate at a final concentration of 1%. Samples were centrifuged (6,000 g) for 10 min at 4 °C. Total protein carbonylation was measured in supernatants using a colorimetric protein carbonyl assay kit in accordance with the manufacturer's instructions (Cayman Chemicals intra-assay coefficient of variation of 4.7%) at 360 nm using a SpectroQuant spectrophotometer (BioTek Instruments Inc. Winooski, VT, USA). All samples were tested in duplicate at room temperature.

Measurement of Malondialdehyde (MDA) for Lipid Peroxidation

Lipid peroxidation was measured by MDA quantitation. Tissue samples (10 mg) were washed in cold PBS, homogenized with a polytron in 303μl of lysis solution (Abcam, San Diego, CA, USA) while on ice and then centrifuged at 13,000 g for 10 min. Supernatants were collected and used to measure malondyaldehyde bound to thiobarbituric acid (TBA) by a colorimetric assay kit (TBARS) according to the manufacturer's instructions (Cayman Chemicals) at 360 nm using a μQuant spectrophotometer (BioTek Instruments Inc. Winooski, VT, USA). All samples were tested in duplicate and measured at 540 nm wavelength at room temperature.

Western blotting

Tissue samples were homogenized with a polytron in 250 μl of lysis buffer (1% Triton X-100, 200 mM Tris, 140 mM NaCl, 2 mM EDTA, and 0.1% sodium dodecyl sulfate) with protease and phosphatase inhibitor cocktails (P2714; Sigma-Aldrich), supplemented with 0.15 mM PMSF, 5 mM Na_3VO_4 and 3 mM NaF, as previously described[22,23,25,26]. Homogenates were sonicated in a tank sonicator (Fisherbrand USA) for 15 min at 4 °C and centrifuged (10,000 g) for 10 min at 4 °C[22]. The total protein content was measured in the supernatant using the Bradford method. A total of 40 μg of protein were loaded onto a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, Hercules, CA, USA)[26], electrotransferred to a PVDF membrane using a Trans-Blot® Semi-Dry system (16 V, 60 min) for low molecular weight proteins, or using a Mini Trans-Blot® Cell system (70 V, 2 h) (Bio-Rad, Hercules, CA, USA) for high molecular weight proteins. The membranes were incubated for 1 h in blocking solution (5% nonfat dry milk in Tris-buffered saline plus 0.1% Tween 20), followed by overnight incubation at 4 °C[26] or 3 h of incubation at room temperature[22] with primary antibodies. Membranes were washed (3 X for 5 min) in Tris-buffered saline plus 0.1% Tween 20 and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:10,000 in blocking solution. The immunoblots were developed using an enhanced chemiluminescence (ECL) detection kit (Amersham-GE, USA). The band densitometric analyses were achieved using the ImageJ software.

Antibodies

Anti-TNFα, COX2, NFκβ, INFγ, IL-1β, IL-5 and IL-6 antibodies were purchased from Cell Signaling Technologies (San Diego, CA) and used as pro-inflammation markers. Anti-IL-10 and IL-11 antibodies were from Santa Cruz BioTech, (Santa Cruz, CA) and used as anti-inflammatory markers. To measure memory surrogates anti-AKT, p-AKT (Ser473), GSK3β, p-GSK3β (Ser9), Amyloid-β (Aβ−42) (Cell Signaling), TREM2, NGF, TAU and p-TAU (Ser396) were obtained from Abcam (San Diego, CA) while GFAP was from Santa Cruz Biotech. and Iba1 antibody was purchased from Abcam (San Diego, CA). Anti-GAPDH, used for signal normalization, as well as anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were from Cell Signaling. (San Diego, CA).

Pro- and anti-inflammatory cytokines in plasma

Blood was draw from the inferior vena cava using a syringe containing 50 mM EDTA pH 8 and centrifuged at 1700 g for 10 min at 4° C. Plasma was recovered and used for Luminex xMAP technology with the Milliplex brand of magnetic-bead fluorescent immunoassay for TNF-α, IFN-γ, IL-1ra IL-15, IL-3 and IL-1β (MilliporeSigma EMD). Plasma samples for the assay were processed according to the manufacturer instructions and evaluated in the Bioplex plate reader (Bio-Rad, Hercules, CA, USA). Concentration of cytokines was calculated using the standard curve and validated using the ProcartaPlex internal controls of the assay.

Amyloid-β (Aβ 42) measurements by ELISA

APP null mouse hippocampus samples were kindly provided by Dr. Hemal Patel. Hippocampus was homogenized in cold buffer of 5 M guanidine-HCl/50 mM Tris, pH 8.0, protease inhibitors and incubated with shaking for 3 h at room temperature and then processed for Aβ 42 assessments according to the manufacturer instructions (Invitrogen ThermoFisher). Homogenized samples were centrifuged at 15000 g for 20 min at 4° C. Supernatants were collected and diluted in cold PBS with protease inhibitors and used for Aβ 42 measurements in a microplate reader (SpectroQuant) at 450 nm wavelength using a SpectroQuant spectrophotometer (BioTek Instruments Inc. Winooski, VT, USA). Aβ 42 concentrations were calculated using the standard curve from the assay.

Caspase 3 and 9 activity measurements

The activity of caspases as surrogates of apoptosis was measured using the caspase-3 and caspase-9 kits both from Abcam. Hippocampal samples were homogenized and processed as per manufacturer instructions. Caspases 3 and 9 recognize DEVD and LEHD sequences, respectively, and cleave the labeled substrate p-NA developing color reaction which was measured in a microplate reader al 405 nm wavelength using a SpectroQuant spectrophotometer (BioTek Instruments Inc. Winooski, VT, USA).

Locomotor activity and Open Field Task (OFT)

To avoid stressful conditions that could affect the performance or consolidation of each task, animals were transported from the vivarium to the experimental room during 3 consecutive days and left in the testing room for 1 h prior starting the test and were left at the same location for an additional 2 h at the end of the test[27]. General locomotor activity and anxiety-like behavioral responses to a novel environment were measured in an OF apparatus, consisting of an acrylic 50 cm (length) X 50 cm (width) surrounded by a 50 cm high wall. Two areas were virtually differentiated in the OF: the periphery (outer zone 10 cm from the wall) and the central area (the rest of the OF) (Fig. 7A). Animals were gently placed in the center of the area at the beginning of the test, allowed to move free and uninterrupted in the quadrants of the OF to explore the environment for 10 min period. Animal movements and paths were recorded by a Sony Cyber Shot video camera (Sony, USA). The video recordings were used to measure total distance traveled and the time spent in each area[28].

Object Recognition Task (ORT)

To avoid behavior alterations due to exposure to a new environment the animals were transported to the testing room as in OFT. Animals were habituated individually to the open empty arena to free exploration for 5 min in two consecutive days. On the following day animals were familiarized during 5 min to two identical aligned objects (A + A) placed in the open field at 10cm from each wall. Then, mice were sent back to their home cages for 1 h and reintroduced to the arena after one object was exchanged (A + B) and allow them to explore for 5 min (short-term memory). After 24 h object B was exchanged for object C and allow them for an exploratory period of 5 min (long-term memory) (Fig.8A). Exploration was considered as pointing the nose toward an object at a distance of less than 1 cm and/or touching it with the nose. Turning around or sitting close to the object was not considered as

exploratory behavior[29]. The trials were video recorded using a Sony Cyber Shot camera (Sony, USA). for subsequent scoring by two different individuals blind to the experimental conditions. Object recognition was defined as time spent between novel (TN) and the time sum of both objects novel and familiar (TF) [(TN)/(TN+TF)].

Data and Statistical Analysis

Results are expressed as mean ± standard error (SEM). Statistical analysis was pursued using two-way analysis of variance followed by a Tukey post-hoc tests and unpaired t-test when applicable using GraphpadPrism version 8.0 (GraphPad Prism, San Diego, CA, USA). Statistical significance was defined when $p < 0.05$.

Results

Protein Carbonylation and MDA Levels

Total protein carbonylation was significantly increased ($p<0.05$) by ~2.5 fold in AC (8.45 ±1.3 nmol/mg of protein) vs. YC (3.25 ±1.1 nmol/mg of protein). Epi treatment reduced protein carbonylation levels in AE animals by ~25% (6.4 ±0.9 nmol/mg of protein) vs. AC ($p<0.05$) (Fig. 1A). Lipid peroxidation as measured by the presence of MDA increased in AC by ~2 fold (3.4 ±0.6 nmol/mg of protein) vs. YC mice (1.7 ±0.3 nmol/mg of protein) ($p<0.05$). Epi treatment also significantly reduced MDA levels by ~30% in AE (2.35 ±0.4 nmol/mg of protein) vs. AC (Fig. 1B).

Pro- and anti-inflammatory endpoints

To investigate the impact of aging and Epi treatment on inflammation, biomarkers were assessed by Western blot in hippocampus (Fig. 2) and using the Milliplex immunoassay system (plasma) (Fig. 3). In hippocampus of AC animals, all pro-inflammatory markers (COX2, TNFα, NFκβ, IFN-γ, IL-1β, IL-5, and IL-6) increased significantly (vs. YC) ranging ~18% (IL-5) to 30% (COX2), while anti-inflammatory IL-10 and IL-11 were decreased (~20 and 30% respectively). In YE animals, treatment was able to significantly decrease IFN-γ protein levels by ~20% vs. YC. In AE animals, treatment significantly reduced the protein levels for all examined molecules (COX2, NFκβ, TNFα, IFN-γ, IL-5, IL-6 and IL-1β) by ~15–20%, while increased IL-10 and IL-11 by ~30% vs. AC group. Similar results were obtained from the Milliplex system assays in plasma of AC animals in all pro-inflammatory markers tested. TNFα, IFN-γ, IL-15, IL-3 and IL-1β, were increased significantly, while anti-inflammatory IL-1ra was decreased (vs. YC) ranging from ~150% for TNFα and IL-15; and up to ~200% for IL-1β, IL-3 and IFN-γ. In AE animals, treatment was able to significantly decrease all the cytokines almost at YC levels with the exception of IFN-γ, which only shown an important decrease trend and significantly increase the IL-1ra.

Tau/β-amyloid pathway related endpoints

Tau hyperphosphorylation, (including its pathway modulators glycogen synthase kinase 3-β [GSK3β] and AKT) (Fig. 4A–C) and soluble β-amyloid (Aβ 42) (Fig. 5A–B) protein levels were measured. In AC animals, p-AKT and p-GSK3β protein levels decreased ~38% and ~47% (vs. YC) respectively (Fig. 4A and B), while hp-tau increased significantly ~90% (Fig. 4C). In young mice, Epi treatment (YE) significantly increased p-AKT and inhibitory

p-Ser9-GSK3β levels to ~110% and ~95% respectively. In AE animal treatment significantly stimulated p-AKT (~40%) and p-GSK3β (~50%), while hp-tau was decreased (~60%) vs. AC. On the other hand, in AC animals, Aβ 42 increased significantly ~150% vs YC. While in AE animal treatment significantly decreased Aβ 42 by ~70% (Fig 5A–B). As a control for the Aβ 42 presence, APP knock out mice were used, and as expected Aβ 42 protein levels were undetectable (Fig 5A–B).

Neuronal viability/health

As an indicator of neuronal health, nerve growth factor (NGF) and TREM2 were measured (Fig. 6A). AC animals showed a ~50% and ~60% decrease in NGF and TREM2 levels respectively (vs. YC). However, Epi treatment significantly recovered NGF, and TREM2 levels in AE (~35%) vs. controls. Contrary glial associated activators GFAP and Iba1 protein levels increased ~30% and ~45% respectively in AC vs YC. (Fig. 6B); while Epi treatment significantly decreased GFAP and Iba1 by ~30% and ~40% respectively. Caspases (3, 9) activity as a surrogate of apoptosis was measured (Fig. 6C). In AC animals, caspase activity was increased (~180%) vs YC. AE animals showed a decreased activity in caspase 3 and 9, (~40% and ~45% respectively); however, such decrement does not reach levels as YC.

OFT and ORT tests

The first important parameter in OFT (Fig. 7A) measurements is the total ambulatory distance in order to remove any confusing activity behavioral in test. Total distance traveled results demonstrated no differences in locomotor activity between control and Epi treated animals (Fig. 7B). OFT, used as an indicator of anxiety-like behavior, demonstrated a significant reduction in the outer zone vs. the center zone time with Epi treatment either in young or aged animals (Fig 7C).

ORT was performed to evaluate short and long-term memory in mice as shown in Fig. 8A. Epi treatment significantly improved the object preference score in young and aged animals vs. controls either in short (Fig. 8C) or long-term memory (Fig. 8D).

Discussion

Unique results provide evidence for the beneficial effects of Epi in alleviating OS, inflammation (including systemic indicators), markers of neurodegeneration and cell survival in the hippocampus leading to improved cognitive performance and anxiety measures in aged mice (Fig. 9). The described Epi effects may thus, partly account for the beneficial impact that cocoa consumption has on measures of brain health in mammals including humans.

It is well accepted that longstanding OS can promote the development of neurodegeneration[30,31]. The aged brain may be particularly susceptible to oxidative damage due to high levels of ROS production[32] and age-associated decline in ROS buffering capacity[33]. In this study, we provide evidence as to the beneficial effects that Epi has on protein and lipid oxidation endpoints both in young and senile mice. It is widely believed that antioxidants may exert beneficial effects on OS and that such actions may be secondary to the direct neutralization of ROS. In a previous publication, we demonstrated that in aged

mice, Epi (at the same doses used in this study) can stimulate in a highly effective manner, frontal cortex protein and activity levels of the ROS catalysis system which is mainly comprised of Nrf2, sirtuin3, glutathione peroxidase, thioredoxin, catalase and superoxide dismutase 2 (SOD2). Furthermore, the levels of buffering systems such a reduced glutathione are also enhanced[22]. Thus, tissues that are exposed to high levels of ROS production when treated with Epi, can more effectively neutralize their reactivity. These results are similar to those reported using green tea epicatechin (EGCG) at 2 mg/kg/day which lowered lipid peroxidation and protein carbonylation levels in whole brain tissue from aged rats where proposed mechanisms were ascribed to the upregulation of SOD and catalase levels[34]. Similarly, studies using quercetin (albeit at higher doses, 50 mg/kg/day) in rats attenuate hippocampus and striatum swimming induced OS[35].

The enhanced local and systemic production and release of pro-inflammatory molecules such as COX2, interferon-γ, TNF-α, IL-1β, IL-5 and IL-6 has been shown to increase as a function of aging and likely contribute to the development of neurodegeneration[21]. Key control elements such as NF-κB and AP-1 appear to play central roles in stimulating the production of such molecules. Published *in vitro* studies have reported on the anti-inflammatory potential of cocoa flavanols via the suppression of molecules such as IL-4 and TGF-β[36]. In fact, pure flavonoids (e.g., quercetin, genistein, hesperetin, epigallocatechin-3-gallate) or enriched-extracts, can reduce the expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and COX-2), down-regulate inflammatory markers and prevent neural damage. Studies using young (3 month old) female mice, have reported on the beneficial effects that Epi (2 weeks at a concentration equivalent to 100 mg/kg/day) has on genes associated with learning, neuroinflammation, neurodegeneration and cell death as per the microarray analysis of hippocampus samples[21]. The beneficial effects of Epi on neuroinflammation and neurodegeneration endpoints are similar to those noted on pro-inflammatory COX2, NFκβ, TNFα, INF-γ, IL-5, IL-6 and IL-1β and anti-inflammatory IL-10, IL-11 and IL-1ra in our study. However, there is a 100-fold difference in the dose used as we only treated animals with 1 mg/kg/day. Other flavonoids such as ECGC have demonstrated to possess anti-inflammatory potential. In a study, using a mouse model of amyotrophic lateral sclerosis treatment with ECGC at 10 mg/kg/day leads to the suppression of NF-kB protein levels in the spinal cord[37]. Quercetin also suppresses proinflammatory gene expression and reduces apoptosis in cocultures of microglial and neuronal cells[38].

According to the “amyloid hypothesis” of AD progression[39], the overproduction or failure of clearance of β-amyloid in the brain leads to its deposition and the progression of pathological events, including the formation of neurofibrillary tangles. Both processes are linked to the hp-tau. Signaling pathways associated with these events include GSK3β which appears to activate tau hyper-phosphorylation leading to the formation of neurofibrillary tangles *in vivo*. Opposite to this, is the inactivation of GSK3β through the PI3-kinase/AKT signaling pathway which is believed to prevent tau hyperphosphorylation and apoptosis[40]. Interestingly, previous studies have linked AKT-induced inhibition of GSK3β as a mechanism for Epi induced anxiolytic effects in young healthy mice following a 14-week treatment with Epi[19]. Our results are in accordance with such reports as Epi treatment significantly improved hippocampus p-AKT/AKT ratios and decreased p-GSK3β/GSK3β ratio in aged mice, demonstrating Epi effects of this pathway along with the decreased levels

of Aβ 42 and hp-tau. A study by Phan et al., 2019, proved that flavonoids completely inhibited fibrillation of Aβ monomers and induced the formation of spherical, unstructured aggregates with less toxic fibrillary species in an in vitro model. Our results are consistent with them, in the extent of the reduced levels of Aβ 42 shown in treated animals; nevertheless could be interesting to evaluate the formation of these so called “unstructured aggregates”[41].

NGF which promotes neuron growth is known to decrease in aged and AD brains, particularly in memory and cognitive-related areas such as the forebrain and hippocampus[42] and is recognized as an important therapeutic target. Flavonoids such as quercetin and Epi have proven to induce neurite outgrowth in PC12 cells[43]. Moreover, studies of the diabetic retina of rats treated with quercetin evidence a significant increase in neurotrophic factors and reduced cytochrome c and caspase-3 activity[44]. Our results are in accordance with the aforementioned data, as NGF protein levels increased in Epi treated mice. TREM2 expression is important in limiting neuronal toxicity during the early stages of amyloid deposition. The absence of TREM2 expression on microglia impairs their capacity to phagocytose cell membrane debris and increases their production of pro-inflammatory cytokines[6] Epi was capable to increase the levels of TREM2 in YE and SE in a significant manner indicating that flavonoids could have a direct role in the expression of TREM2 during neuroinflammation processes. More work needs to be done in that regard.

GFAP protein and Iba-1 are specific markers for activated astrocytes and microglia, respectively. Quercetin shows a strong anti-inflammatory action by suppressing activated astrocytosis and microgliosis[45]. Since our results showed decreased levels of GFAP and Iba-1 as seen in[45] we can state that Epi treated groups have a suppressed activation of glial cells, therefore reducing antiinflamtory endpoints promoted by age.

Flavonoids have been observed to block oxidative-induced neuronal damage by preventing the activation of caspase-3, providing evidence in support of their potent anti-apoptotic action. In particular, Epi and 3-O-methyl-epicatechin also protect neurons against oxidative damage via a mechanism involving the suppression of JNK, and downstream partners, cjun and pro-caspase-3[16]. Our results also show a decrease in Caspase 3 and Caspase 9 when animals were treated with Epi, promoting as is, neuronal cell survival even during aging. The loss of memory and increase in anxiety are hallmarks of the aging process and onset of neurodegenerative diseases and follow the pathophysiological changes described above including OS, neuroinflammation/degeneration, formation of β-amyloid plaques, hp-tau and formation of tangles. Evidence in humans has linked cocoa consumption to improved cognitive performance as well as increased cortical blood flow[11]. Published studies indicate that Epi, enhances mouse retention of spatial memory in water maze tests and the effects can be reinforced by the combination with exercise. Other flavonoids appear to exert similar effects. Two-month-old mice treated for 2 months with ECGC at 20 mg/kg/day increased proliferation labeled cells in the dentate gyrus leading to improved spatial recognition in mice[46]. In our model, Epi treatment significantly improved anxiety-like behavior as measured in OFT in aged animals, demonstrating a significant reduction in the outer zone vs. center zone time ratio. Also, in the ORT, Epi improved the object preference/recognition score, as a measure of short and long-term memory. Thus, Epi appears to have a role in

preserving short and long-term memory while decreasing anxiety-like behavior levels as seen with aging.

Study results support a neuroprotective potential for the use of low dose Epi in the setting of aging induced brain function decline. Such effects can be attributed to the potential of the flavanol to effectively mitigate OS, inflammation and promoters of brain degeneration while encouraging those associated with neuron growth leading to functional improvements. Noteworthy, is that effects can also be noted in young animals thus, potentially promoting the preservation of brain health. The clinical relevance of such observations needs to be explored in properly designed clinical trials.

Acknowledgments

We kindly appreciate the technical support of Dr. T. Neri and Dr G. Manjarrez

Funding

This work was supported by NIH (DK98717, AG47326) and Department of Defense (PR150090) to Dr. Villarreal and Consejo Nacional de Ciencia y Tecnologia (CONACyT) 253769 to Dr. Ceballos and Instituto Politecnico Nacional (SIP-IPN 20180897) and CONACyT 283938 to Dr. Ramirez-Sanchez

Abbreviations

TNFα	tumor necrosis factor α
COX2	cyclooxygenase 2
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
IL	interleukin
AKT	protein kinase B
p-AKT	phosphorylated protein kinase B
AD	Alzheimer’s Disease
PHF	paired helical filaments
OXPHOS	oxidative phosphorylation
ATP	adenosine triphosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
p-GSK3β	phosphorylated glycogen synthase kinase 3 beta
ROS	reactive oxygen species
CNS	central nervous system
NFT	neurofibrillar tangles

OS	oxidative stress
Epi	(−)-epicatechin
MDA	Malondialdehyde
TBA	thiobarbituric acid
PVDF	polyvinylidene difluoride
HRP	horseradish peroxidase
NGF	Nerve Growth Factor
EDTA	ethylenediaminetetraacetic acid
TBA	thiobarbituric acid
TREM2	triggerin receptor expressed on myeloid cells2
Cas	caspase
IFN-γ	interferon gamma
β-A	beta amyloid
GFAP	glial fibrillary acidic protein
WB	Western blot
PMSF	phenylmethane sulfonyl fluoride
ECL	enhanced chemiluminescence
OFT	Open Field Task
ORT	Object Recognition Task
AU	arbitrary units
Aβ	amyloid beta
MAP	microtubule-associated protein
IL-1ra	interleukin 1 receptor antagonist
Iba1	ionized calcium-binding protein or allograft inflammatory factor 1

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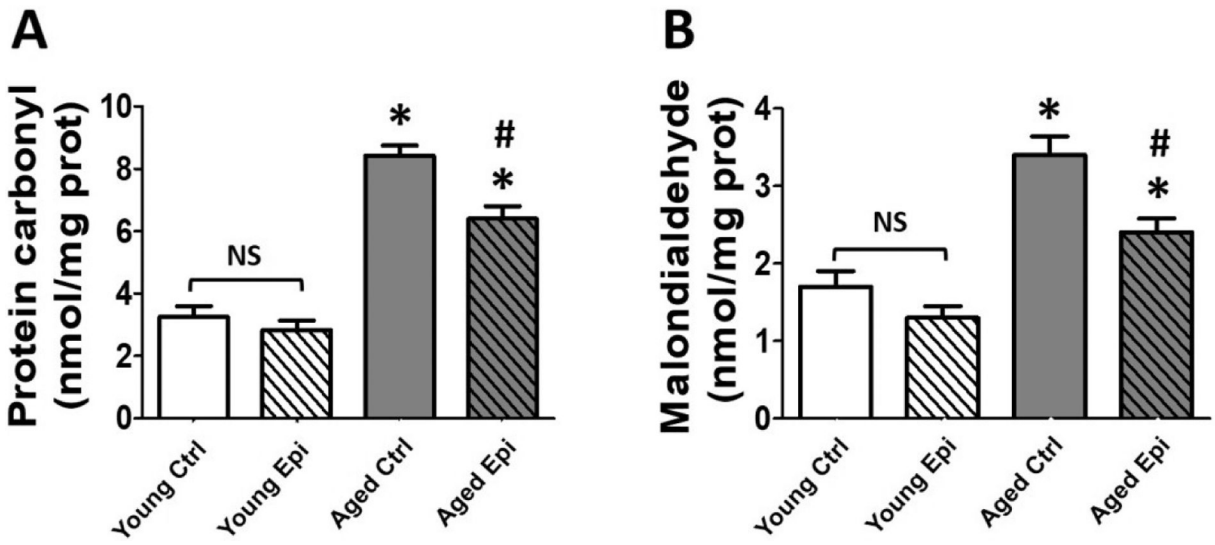

Figure 1.

Oxidative stress indicators level in the hippocampus of young and aged control (YC and AC) and epicatechin treated mice (YE and AE). (A) Changes observed in total protein carbonylation levels. (B) Changes observed in malondialdehyde-bound thiobarbituric acid (MDA) levels. Values were normalized by sample protein content (n = 7/ group, *p < 0.05 vs. YC, #p < 0.05 vs. AC).

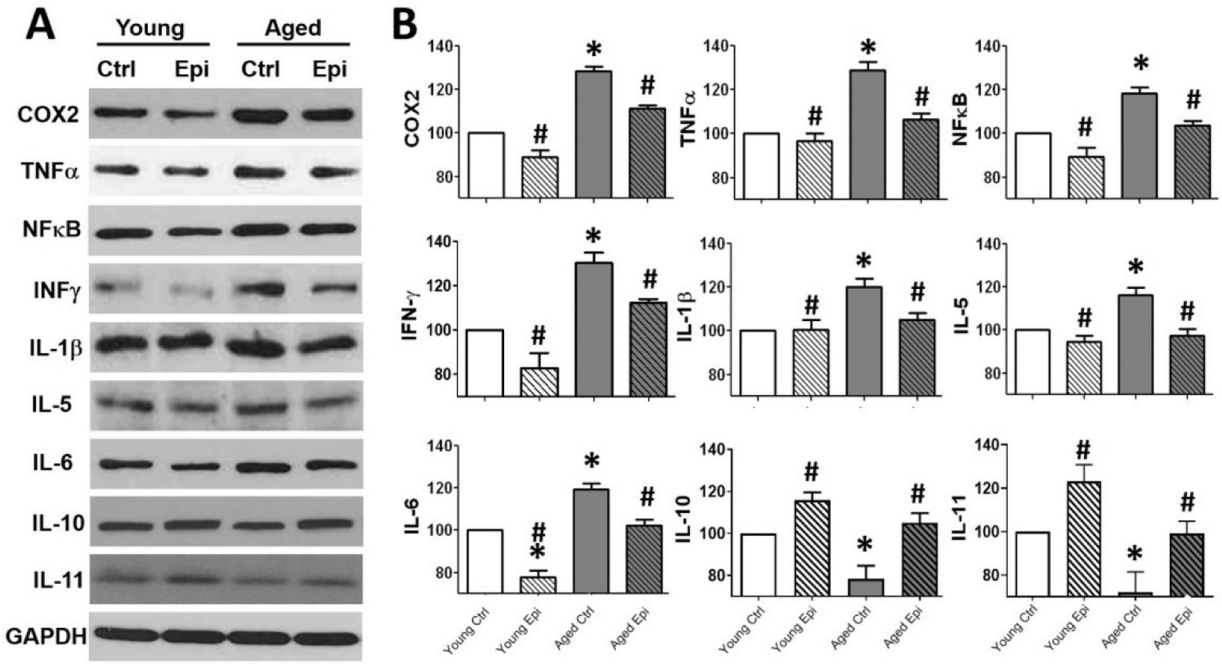

Figure 2.

Inflammatory modulators and Interleukin(s) relative protein levels in the hippocampus of young and aged control (YC and AC) and epicatechin treated mice (YE and AE). (A) Representative Western blot images observed in COX2, TNF-α, NFκB, INF-γ, IL-1β, IL-5, IL-6, IL-10 and IL-11 protein levels. (B) Relative changes noted with YC values set as 100%. Protein levels were normalized using GAPDH values (n = 7/ group, *p < 0.005 vs. YC, #p < 0.05 vs. AC).

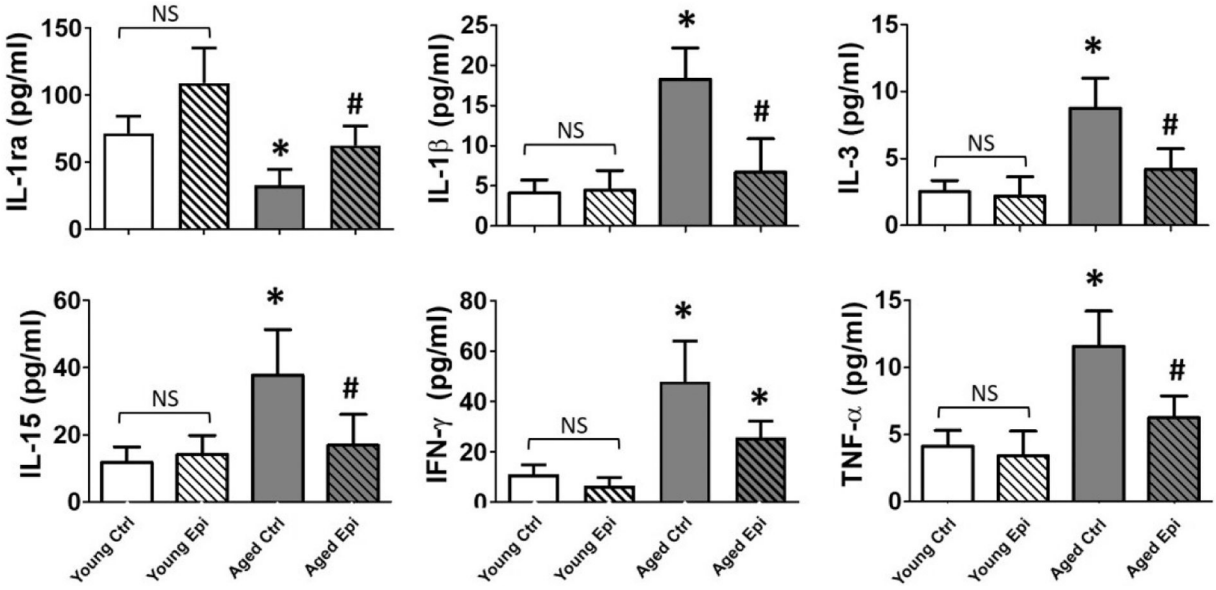

Figure 3.

Pro and anti-inflammatory cytokines levels in plasma. Quantification of IL-1ra, IL-1β, IL-3, IL-15, IFN-γ and TNF-α plasma levels in young and aged control (YC and AC) and epicatechin treated mice (YE and AE) values (n = 7/ group, *p < 0.005 vs. YC, #p < 0.05 vs. AC).

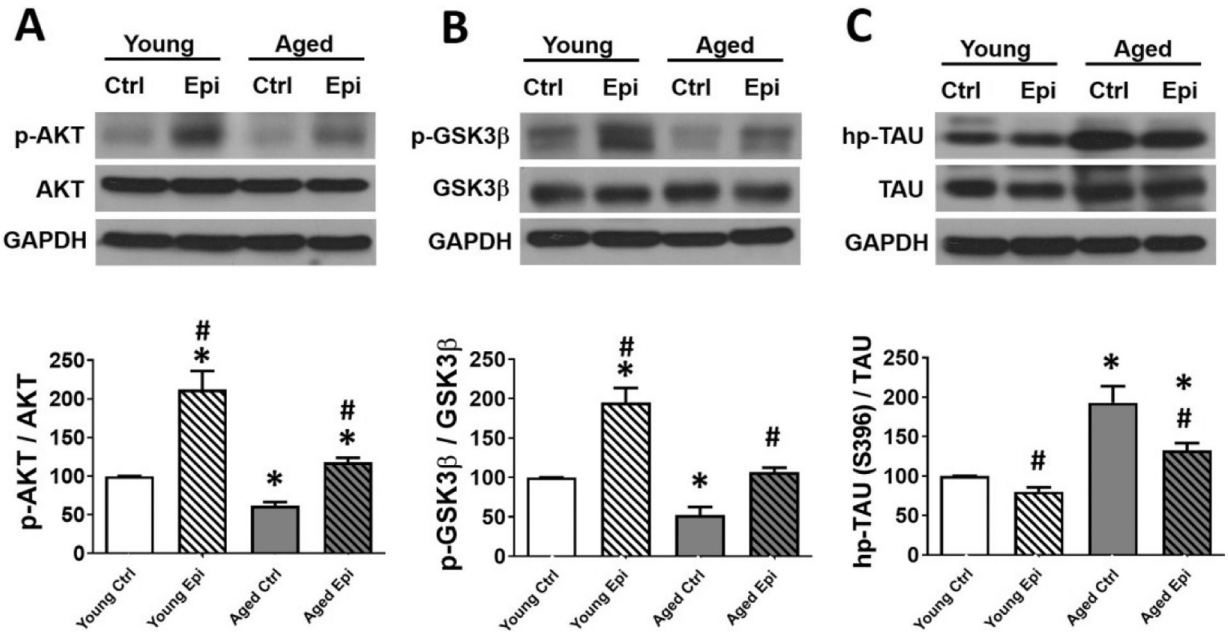

Figure 4.

AKT, GSK3β and TAU total and phosphorylated relative protein levels in the hippocampus of young and aged control (YC and AC) and epicatechin treated mice (YE and AE). (A) Representative Western blot images observed in p-AKT (A), p-GSK3β (B) and hp-Tau (C). Protein levels are reported relative to total protein with YC values set as 100%. Protein levels were normalized using GAPDH values (n = 7/ group, *p < 0.05 vs. YC, #p < 0.05 vs. AC).

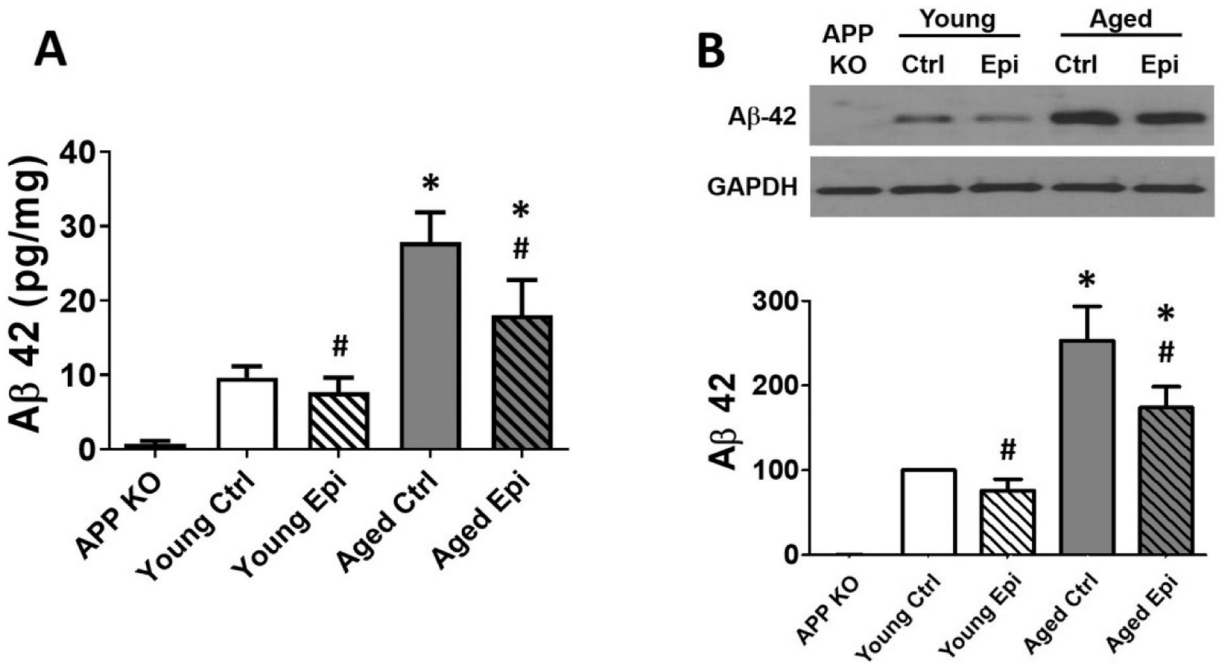

Figure 5.

Aβ 42 protein levels of young and aged control (YC and AC) and epicatechin treated mice (YE and AE). (A) Aβ 42 protein levels in plasma samples by ELISA reported as pg of Aβ 42/mg of total protein. (B) Representative Western blot images and relative changes noted with YC values set as 100%. Protein levels were normalized using GAPDH values (n = 7/group, *p < 0.005 vs. YC, #p < 0.05 vs. AC).

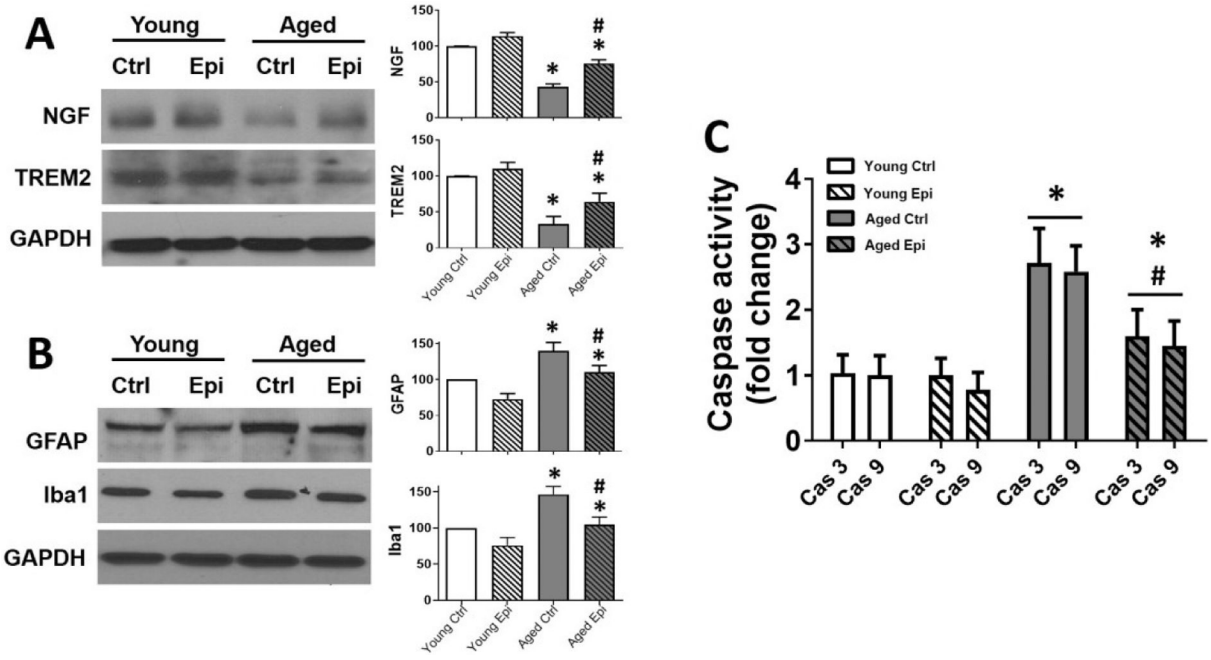

Figure 6.

Protein levels of NGF, TREM2, GFAP and Iba1 and Caspase activity in hippocampus of young and aged control (YC and AC), and epicatechin treated mice (YE and AE). Representative Western blot images observed, for NGF, TREM2 (A) and GFAP and Iba1 (B). Relative changes noted with YC values set as 100%. Protein levels were normalized using GAPDH values. Caspase (Cas) 3 and 9 activity is reported as fold change (C) values (n = 7/ group, *p < 0.05 vs. YC, #p < 0.05 vs. AC).

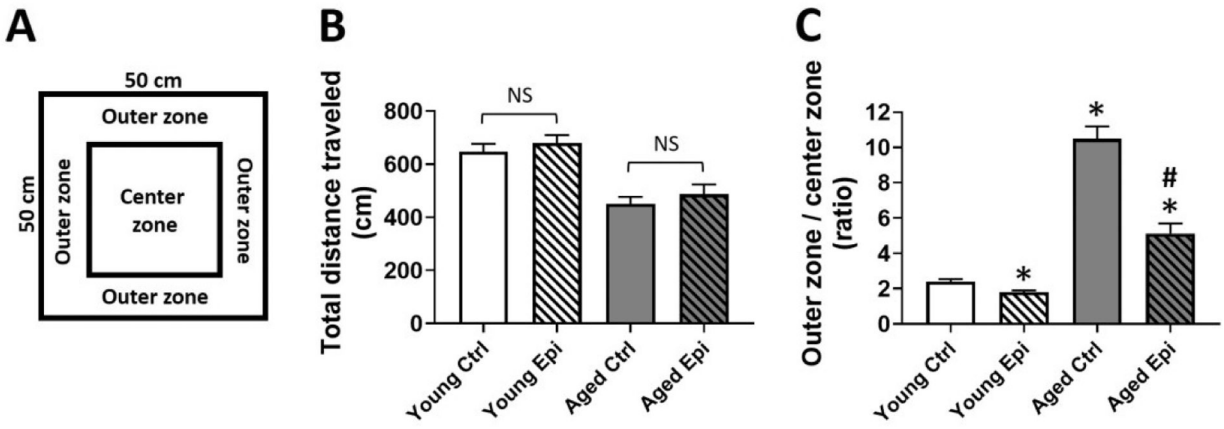

Figure 7.

Open field testing of young and aged animals treated with vehicle (control) or epicatechin for 4 weeks. (A) Schematic representation of open field test. (B) Total distance traveled (in cm) in the open field. (C) outer zone vs centrer zone ratio of young and aged control (YC and AC) and epicatechin treated mice (YE and AE). Results are reported as outer vs center time in seconds (s) ratio. (n=7/group, *p<0.05 vs. YC), #p < 0.05 vs. AC).

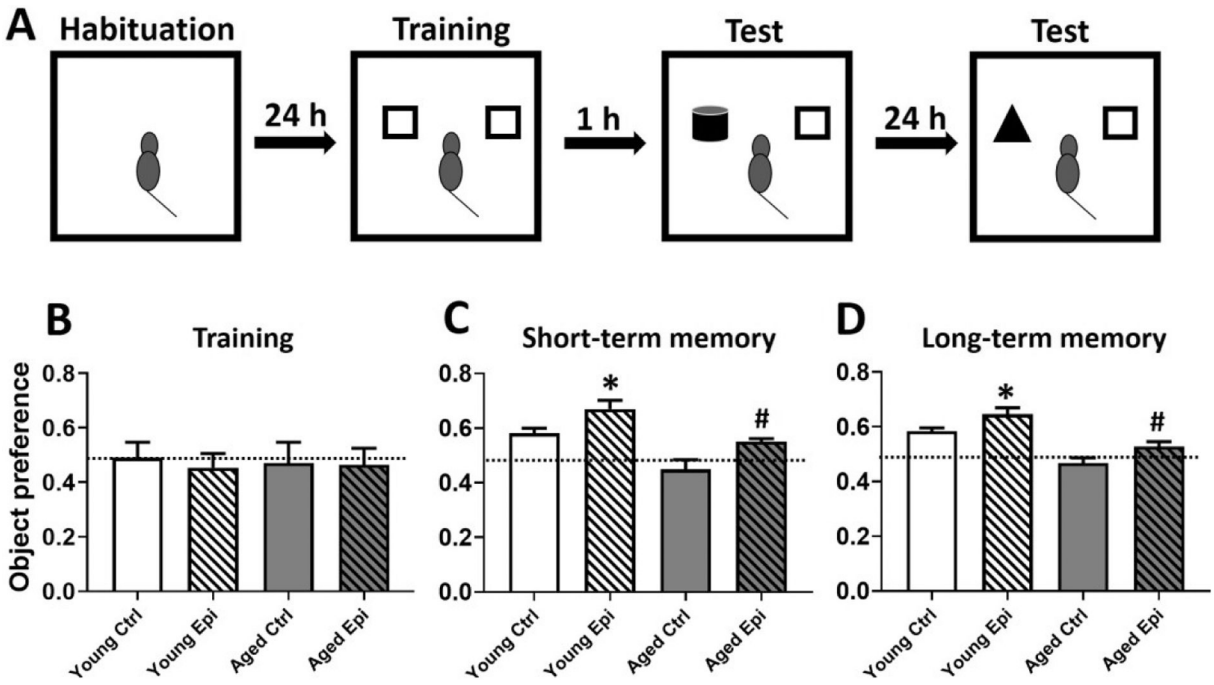

Figure 8.

Object recognition test of aged animals treated with vehicle or epicatechin for 4 weeks. Schematic representation of the object recognition test (A). Graphic representation of the object preference score during the training (B), during the short-term memory (1h) test (C) and during the long-term memory (24h) test (D). Values compare chance score of the object preference (0.5) vs. those recorded at day 28. (B) Discrimination index obtained from 28th recorded values divided by baseline values. Results are reported as latency time in seconds (s). (n=7/group, *p<0.05 vs. before, #p < 0.05 vs. AC).

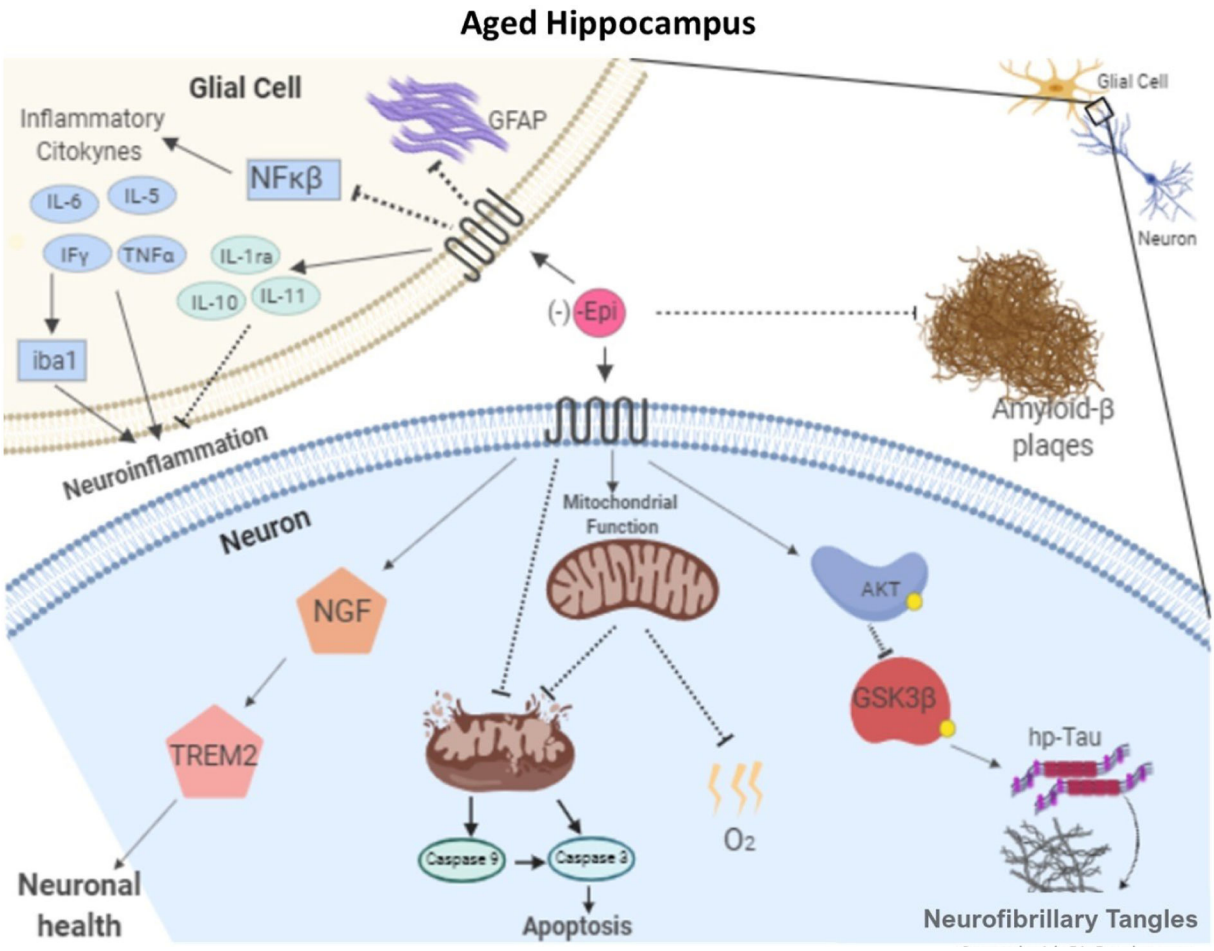

Figure 9.

Schematic representation of proposed mechanisms for which epicatechin reduces neuroinflammation and Tau-hp, improving the noted endpoints such as memory and learning.