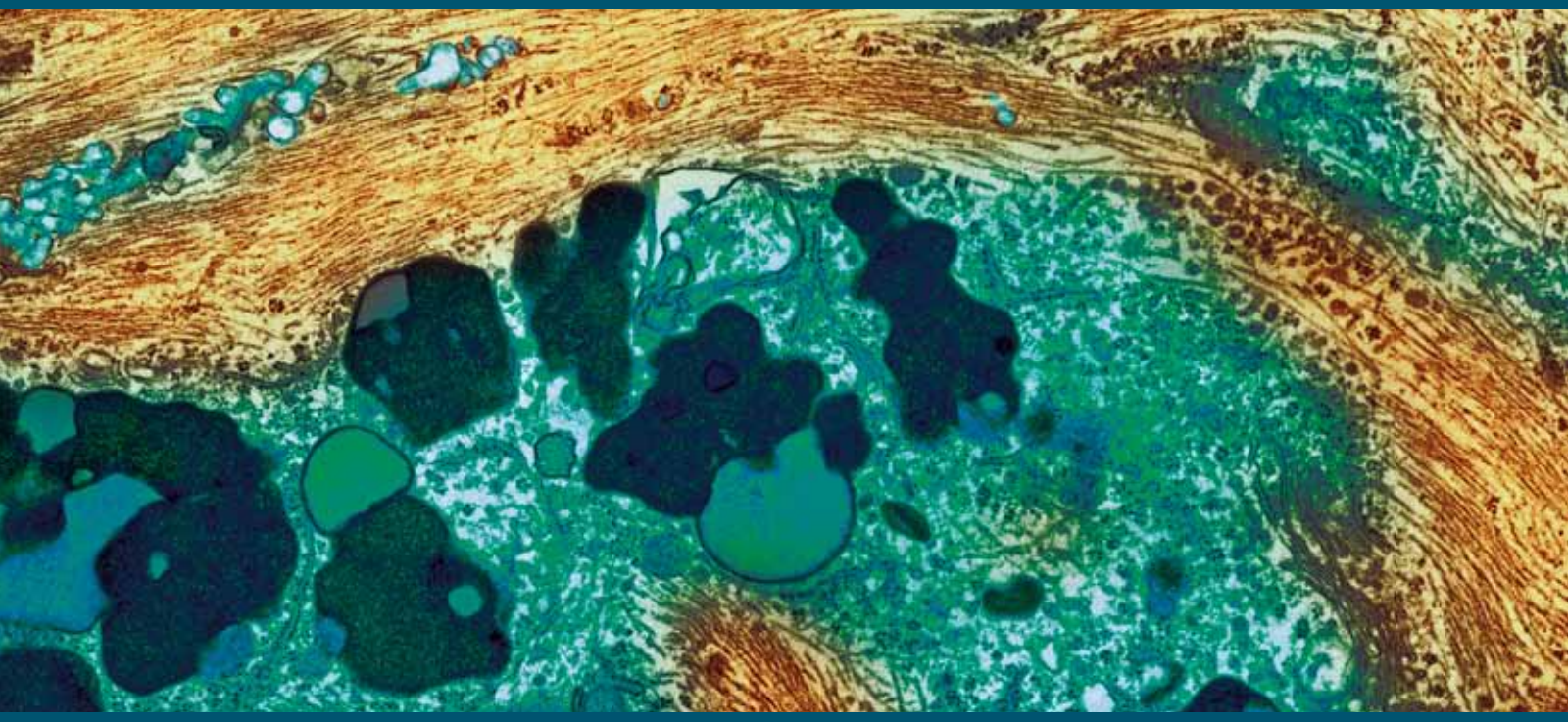
International Journal of Alzheimer's Disease

# Neuroprotection and Neuroregeneration in Alzheimer's Disease

Guest Editors: Kiminobu Sugaya, Moussa B. H. Youdim, Agneta Nordberg, and K. S. Jagannatha Rao



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# Editorial Board

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*Editorial*

# Neuroprotection and Neuroregeneration in Alzheimer's Disease

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Neurodegeneration in Alzheimer's disease (AD) is thought to be initiated by a cascade of neurotoxic events that include oxidative stress, brain iron dysregulation, glutamate excitotoxicity, nitric oxide, inflammatory process, neurotoxic processing resulting from misfolding, and aggregation of Abeta peptide, as a possible consequence of the demise of ubiquitin-proteasome system (UPS) which is demonstrated neurochemically and by transcriptomics and proteomic profiling. AD is benefitted from the symptomatic effects of cholinesterase inhibitors and glutamate antagonist (memantine), which act on a single molecular target. Such drugs have limited symptomatic activities, and current pharmacological approaches have severe limitations in their ability to be neuroprotective and to modify the course of the disease, offering incomplete and transient benefit to patients. Yet in laboratory and animal models, a number of drugs have demonstrated the ability to be neuroprotective, but in clinical trials, they have failed as a form of symptomatic treatment and disease modification. This situation is not different from that of Parkinson's disease or amyotrophic lateral sclerosis, where the same problems exist. There are a number of valid reasons why we have failed to alter the course of these progressive neurodegenerative disorders. First and foremost, the models employed *in vitro* and *in vivo* are not true representations of complex disease as seen in man. Most of the effort has been in the direction of preventing the formation and overexpression of Abeta peptide in transgenic mice expressing Abeta peptide and plaques. Yet in these animals, there is no process of neurodegeneration. Yet one must question whether the disease is a disorder of Abeta-peptide-induced plaque formation resulting in the cognitive decline or if other processes are involved. The hope is that the newly developed rat transgenic model, which emulates many features of AD, will advance the pathological understanding of the disease and may lead to the development of new therapeutic strategies. The complex pathology of AD pathways includes changes in gene expression, protein metabolisms, response of receptors, level of neurotransmitters, activity of kinase, and signaling pathways. The most important events in neuroprotection and neuroregeneration are the selection of drugs that include synthetic products, natural products, amyloid synthesis, hormonal balance, and nanoparticles intended for a variety of biochemical targets such as oxidative stress. This special issue provides a new knowledge based on therapeutic candidates designed to act on multiple neural and biochemical targets involved in the neurodegenerative process and to possess neuroprotective and neurorestorative activities.

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*Research Article*

# Alterations in Lipid Levels of Mitochondrial Membranes Induced by Amyloid-$\beta$: A Protective Role of Melatonin

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Alzheimer pathogenesis involves mitochondrial dysfunction, which is closely related to amyloid-$\beta$ (A$\beta$) generation, abnormal tau phosphorylation, oxidative stress, and apoptosis. Alterations in membranal components, including cholesterol and fatty acids, their characteristics, disposition, and distribution along the membranes, have been studied as evidence of cell membrane alterations in AD brain. The majority of these studies have been focused on the cytoplasmic membrane; meanwhile the mitochondrial membranes have been less explored. In this work, we studied lipids and mitochondrial membranes *in vivo*, following intracerebral injection of fibrillar amyloid-$\beta$ (A$\beta$). The purpose was to determine how A$\beta$ may be responsible for beginning of a vicious cycle where oxidative stress and alterations in cholesterol, lipids and fatty acids, feed back on each other to cause mitochondrial dysfunction. We observed changes in mitochondrial membrane lipids, and fatty acids, following intracerebral injection of fibrillar A$\beta$ in aged Wistar rats. Melatonin, a well-known antioxidant and neuroimmunomodulator indoleamine, reversed some of these alterations and protected mitochondrial membranes from obvious damage. Additionally, melatonin increased the levels of linolenic and n-3 eicosapentaenoic acid, in the same site where amyloid $\beta$ was injected, favoring an endogenous anti-inflammatory pathway.

## 1. Introduction

We hypothesized that due to its amphipathic nature [1], its physicochemical functions [2] and aided by the induced oxidative stress [3, 4], A$\beta$ paves its own pathway from the extracellular space to the mitochondria where it disrupts membrane fluidity and causes energetic dysfunction. This mechanism of membrane permeabilization induced by A$\beta$ and its own internalization might be the major cause of mitochondrial dysfunction.

Following injection of A$\beta_{1-42}$ into the hippocampus of healthy Wistar 6-month-old rats, we reported deposits of this peptide fragment forming plaques in the extracellular space. Thereafter A$\beta$ was observed in cytoplasmic membranes, especially those of axons, where it accumulated in one or two poles of the axons giving the appearance of onion bulbs, as observed in demyelinating pathologies. Demyelination is also a feature of Alzheimer's disease (AD) [5, 6]. It is noteworthy that the animals used in the experiments had no other condition or genetic predisposition to form plaques or other AD features. A$\beta$ peptide then appeared in the cytoplasm, and finally in mitochondrial membranes, where its presence was associated with mitochondrial dysfunction. The question remaining is what alterations A$\beta$ produce in the

lipid composition of mitochondrial membranes, particularly in those fatty acids and phospholipids previously related to the A$\beta$ pathogeny. Moreover, if A$\beta$-induced oxidative stress plays a key role in damaging lipids in cell membranes, what is the potential role of the antioxidant melatonin when this process affects mitochondrial membranes?

Lipid and fatty acid changes have been studied primarily in plasma membranes or in synaptosomes prepared from AD postmortem brains; those changes have been associated with aging, A$\beta$ deposits, dementia, and even with mild cognitive impairment. Other results have been obtained using purified synaptosomal plasma membranes from transgenic mouse brain. To our knowledge, no studies have focused on mitochondrial membranes in response to *in vivo* extracellular deposits of fibrillar A$\beta$. This approach has relevance to the aforementioned hypothesis and our previous results.

The lipid sensing and lipid-regulated proteolysis is a well-characterized phenomenon. This involves the processing of the sterol regulatory element binding proteins (SREBPs), as the best known example [7]. Likewise, saturated fatty acids are related both to amyloidogenesis and to tau hyperphosphorylation. Palmitic acid (PA) is related to the $\beta$-site amyloid precursor protein (APP) cleaving enzyme (BACE1) upregulation and amyloidogenic processing of APP in primary rat cortical neurons [8]. Also, cortical neurons growing in conditioned media from astrocytes treated with palmitic acid and the unsaturated oleic acid expressed hyperphosphorylation of the tau protein. This was later found to be an oxidative-related phenomenon since the addition of the antioxidant N-acetyl cysteine reduced hyperphosphorylation of tau [9]. Changes or reaccommodations of fatty acids within the lipidic structures are continuous, and this may represent oxidative stress-induced remodeling [10]. However, an AD-specific or an A$\beta$-induced specific change does not exist. Nonetheless, it is also known that unsaturated fatty acids, specifically oleic and linoleic acids, may stimulate presenilin-1 levels and $\gamma$-secretase activity, which participate in the generation of A$\beta$ [11].

The relationship between oxidative stress, peroxidation of membrane lipids, and neurodegeneration has been primarily explored in postmortem studies, where a significant decline in polyunsaturated fatty acids (PUFAs), especially arachidonic and docosahexenoic acids, has been found [12]. These changes are directly related to the augmentation in 4-hydroxynonenal, a toxic by-product of the peroxidation of membrane PUFAs, especially arachidonic acid (AA) [13]. In fact, A$\beta$ may induce the phospholipase A2, responsible for AA release from cytoplasmic membranes [14]. The observed drop in docosahexaenoic acid (DHA), on the other hand, is correlated with the dietary intake of DHA which may reduce the amyloid burden [15] by stimulating the non-amyloidogenic processing of APP [16]. Fatty acids modulate the production and activity of a variety of neurotransmitters and the alterations of fatty acids in the diet of rodents have been demonstrated to result in changes in the ability of the animals to learn or retain new information [17, 18]. The ratio between unsaturated and saturated FA (U/S) expresses the degree of unsaturation being linked to less membrane fluidity as an adaptative phenomenon. Changes in the ratio of these fatty acids have been thought to be involved in a variety of diseases including cancer, diabetes, and neurologic diseases. The overproduction of A$\beta$ leads to a decline in $\Delta$-9 desaturase activity with an alteration in membrane fatty acids This results in altered membrane mobility leading to a decline in neurotransmitter activity and a decreased release of acetylcholine [19].

It has been demonstrated that A$\beta$ peptides interact with anionic lipids which leads to a significant alteration in the properties of the bilayer itself. Phosphate groups in anionic lipids and aliphatic aminoacids (Val-Val-Ile-Ala) at the C-terminal end of A$\beta$ mediate that interaction while oxidative stress induces a significant rise in anionic phosphatidylserine (Ptd-Ser). Membrane disruption induced by A$\beta$-peptide is mediated through perturbations of the lipid order caused by interaction of peptides with head groups and/or formation of micelles [20]. Reciprocally, when incubated with Ptd-Ser, A$\beta$ undergoes transformation from a random coil to a $\beta$-structure [21]. Furthermore, there seems to be a cell selective neurotoxicity due to A$\beta$ determined by surface Ptd-Ser, apart from the levels of ATP [22]; this also relates to our hypothesis. Thus, the capacity of cells to bind A$\beta$ seems to be associated with cells with expressed measurable Ptd-Ser on the membrane surface, and this feature is correlated with apoptotic signaling. It should be noted that Ptd-Ser is normally found on the inner face of the surface membrane of healthy cells. In the early stage of apoptosis, however, or under specific stimulation, such as $Ca^{2+}$ influx, a hallmark in the mitochondrial pathogenic mechanisms possibly involved in AD [23, 24], Ptd-Ser can translocate to the outer leaflets of the plasma membrane and be exposed to the extracellular space. Thus, Ptd-Ser becomes a surface receptor site for A$\beta$ binding, in such a manner that annexin or apolipoprotein E2, proteins with the ability to interact with Ptd-Ser, may protect neurons from A$\beta$ neurotoxicity [25]. Since Ptd-Ser is an anionic phospholipid, it may produce an acidic local environment, which is optimal for aggregation of the A$\beta$ peptide [26].

Phosphatidylcholine (PtdChol) is a major constituent of cell membranes, commonly found in the outer leaflet, and it is a particular target for A$\beta$. For example, evaluated in zwitterionic bilayers based on PtdChol membranes, A$\beta$ associates with lipid heads, and when fused into a zwitterionic planar bilayer, it is rapidly transformed from helical- to $\beta$-structure and exhibits a channel-like behavior [27]. In this manner, A$\beta$ disturbs intracellular calcium homeostasis because it renders lipid bilayers permeable to ions.

Changes in packing and orientation of phospholipids in membranes is a phenomenon promoted by cholesterol, which modulates the membrane binding of amyloidogenic proteins [28, 29]. It has been documented that cholesterol increases the binding of A$\beta$ to lipid membranes [30, 31]. The concentration of cholesterol in the membranes has been related to the extent and depth of insertion of A$\beta$ into the membrane [32]. However, a computational study using PtdChol and PtdChol/cholesterol bilayers, which mimic the cholesterol depleted and enriched lipid domains of neuronal membranes, revealed a protective role of cholesterol in preventing both A$\beta$-induced

membrane disruption and membrane surface-induced $\beta$-sheet formation [33]. Nonetheless, by electron paramagnetic resonance spectroscopy, it has been demonstrated how, driven by hydrophobic interactions, A$\beta$ is inserted into bilayers, between the outer part of the hydrophobic core and the external hydrophilic layer. This causes displacement of cholesterol towards the more external part of the membrane where the crowding of cholesterol in turn causes membrane rigidity in this region of the bilayer [34]. This membrane rigidity has been demonstrated in mitochondria obtained from postmortem AD brain [35]; importantly, alterations in mitochondrial membrane fluidity are primarily related to lipid peroxidation [36], which, again, emphasizes the importance of oxidative stress.

Since oxidative stress is a major event in AD progression and is especially related to membrane dysfunction, mitochondrial failure, and apoptosis, the antioxidant melatonin has proven useful in delaying the progression of damage in AD [24]. We have demonstrated in *in vivo* experiments after the injection of A$\beta$ directly into the hippocampus that orally administered melatonin is more effective in reducing oxidative stress than are vitamins C and E [4]. The effect of melatonin has been shown to be especially protective for PUFAs during nonenzymatic lipid peroxidation [37], as observed in transgenic mouse model of AD [38]. Melatonin also may preserve arachidonic and docosapentaenoic acids as observed during ascorbate-$Fe^{++}$ peroxidation in rat testicular microsomes and mitochondria [39].

The current work is based in those fatty acids or lipids previously reported to be involved in A$\beta$-lipid interactions and the protective effect of melatonin on A$\beta$-induced membrane disruption; this latter process is mediated through perturbations of the lipid order caused by an interaction of peptides with head groups and/or formation of micelles [20]. Our results correspond exclusively at the region of the hippocampus where the A$\beta$ was injected.

## 2. Materials and Methods

*2.1. Animals and Experimental Design.* Surgical and animal care procedures were performed with strict adherence to the guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication number 86–23, Bethesda, MD, USA). All protocols and procedures were approved by the institution's Animal Care and Use Committee. Male Wistar rats (250–280 grams; 3-month-old) were housed in pairs in a colony room on a 12:12 dark/light cycle with lights off at 20:00 h; food and water were provided ad libitum. The rats were divided ($n = 5$) into the following groups: (1) PBS-injected rats, (2) fibrillar A$\beta_{1-42}$-injected rats (fA$\beta$), and (3) $H_2O_2$ (200 $\mu$M) intracerebrally injected rats. Two additional groups, fA$\beta$+Mel and $H_2O_2$+Mel, were included. In this case, the fA$\beta$ or $H_2O_2$-intracerebrally injected animals received antioxidant treatment with melatonin (Sigma, St. Louis, MO, USA) dissolved in the drinking water to yield an estimated daily dose of 20 mg/kg/day. IPBS was used as control instead of A$\beta$ peptides since even nontoxic A$\beta$ derivatives, such as the scrambled A$\beta$ usually employed as control in *in vivo* models, may themselves produce free radicals [40, 41]. $H_2O_2$ was chosen as a positive control because of its close relationship with A$\beta$ pathogeny [42]. $H_2O_2$ is considered its principal mediator [43] and secondary messenger of death signals [44]. Additionally, $H_2O_2$ accumulates in mitochondria long before the appearance of A$\beta$ plaques in the extracellular space as evaluated in Tg2576 mice [45].

*2.2. Brain Coordinates for Hippocampal Injections.* Hippocampal injections of A$\beta_{1-42}$ (2 $\mu$L at a final concentration of 1 mM) were performed as previously described [4, 46, 47]. Lyophilized synthetic A$\beta_{1-42}$ (Sigma, St. Louis, MO, USA) peptide was solubilized ($10^{-4}$ M) in filtered PBS; it was then allowed to incubate with continuous agitation (Teflon stir bar at 800 rpm) at 23°C for 36 h in order to form fibrillar aggregates. Rats, anaesthetized with chloral hydrate (350 mg/kg, i.p.), were placed in a stereotaxic instrument for intracerebral injection over a 5 min period (coordinates: anterior-posterior = −3.8 mm, medial-lateral = 2.0 mm, dorsal-ventral = 2.6 mm from bregma; this corresponds to the CA1 region as determined by the atlas of Paxinos and Watson [48] as a guide) using 5 $\mu$L Hamilton microsyringe coupled to a 30-gauge needle through flexible tubing. The needle was left in place for 5 min after the injection. The same coordinates were used for experiments with $H_2O_2$. 36 hours after the injections, rats were deeply anesthetized and transcardially perfused with 200 mL of PBS. Those animals used for immunohistochemical procedures were additionally perfused with 4% paraformaldehyde. The rats were sacrificed by decapitation and the brain was removed immediately, placed in cold PBS, and a piece of tissue (164–180 mg), including the lesioned area, was taken with a punch (diameter 10 mm), at the base of the needle tract. This piece included the hippocampus and adjacent cortical areas.

*2.3. Immunoelectron Microscopy.* For immunoelectron microscopy, the hippocampal tissue samples were fixed in 4% paraformaldehyde for 24 hours and immersed in 2.3 M sucrose solution for 24 hours. Thereafter, small blocks were cut and postfixed in osmium tetroxide (2% in PB 0.2 M) for 45 minutes and then embedded for 48 hours in Embed 812 (Electron microscopy Sciences). Ultrathin sections of 70–90 nm were cut with an ultramicrotome (Reichert Om3) and mounted on nickel grids and then incubated for 2 hours in 5% BSA and 0.1% fish gelatin. For the immunolabeling experiment, the mounted sections were then incubated for 24 hours at 4°C with the primary polyclonal antibody Anti-$\beta$A (Anti-$\beta$A42, from Santa Cruz) at a dilution 1 : 1000, then washed four times with PBS 0.1 M and 0.1% tween-20, and further incubated for 3 hours at room temperature with a 6 nM gold-conjugated secondary goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) at a dilution of 1 : 500. After four washes with PBS, sections were counterstained with uranyl acetate (2%) for 15 minutes and lead citrate for 5 minutes and examined in a Zeiss EM 906 transmission electron microscope (Oberkochen, Germany).

*2.4. In Vivo Analysis of Mitochondrial Free Radicals.* Analysis of mitochondrial free radical generation-Mitotracker red CM-H2XRos (Molecular Probes), a rosamine derivative used to detect mitochondrial free radicals, was diluted in DMSO to form a 1 mM stock solution. 100 $\mu$L of that solution was diluted in 5 mL of physiological saline and stored sterile at 4°C as a working solution. Applied at a dose of 0.030 $\mu$g/kg, CM-H2XRos did not affect the functional properties of mitochondria after loading, since neither the respiratory output nor cell viability was significantly changed, as evaluated in a separate study (data not shown). Two hours following the intraperitoneal injection of CM-H2XRos, animals were perfused transcardially with PBS followed by 4% paraformaldehyde. The brain was immediately removed and immersed in the fixative for 8–10 h. Following a brief washing in PBS, brain slices were cut into 25–30 $\mu$m thick sections, including the area of interest, with the vibratome and incubated free-floating in Mito Tracker Green (Molecular Probes, Ex/Em 490/516 nm), which selectively stains mitochondria both in live cells and in cells that have been fixed. Then sections were mounted on adhesive (Vecta Bond) coated glass slides, with a DNA dye, 4′,6 diamidino-2-phenylindole (DAPI), containing mounting medium (Vectashield, Vector Laboratories) in order to evaluate mitochondrial mass in cells with nuclear counterstaining in blue (Ex/Em 359/461 nm). The mitochondrial free radicals were analyzed by monitoring the oxidized fluorescence product (Ex/Em 554/576 nm) of CM-H2XRos under a fluorescence microscope. Integrated optical density (IOD), number of mitochondria, and its mitochondrial area were determined by using image analysis software (Image-Pro Plus v5.0). Results are presented here as a CMH2XRos/MitGreen IODs quotient.

*2.5. Mitochondrial Isolation.* For mitochondrial isolation, briefly, brain tissue was minced and placed in prechilled Dounce homogenizer with SHE buffer (0.25 M sucrose, 5 mM HEPES and 1 mM EGTA,. PH 7.4), followed by centrifugation at 2,500 rpm for 10 min, 4°C, and recentrifugation of the supernatant (8,500 rpm, 10 min), to obtain a crude mitochondrial pellet. Following a 10 min incubation in ice, the pellet was resuspended again in SHE plus delipidized bovine serum albumin (Sigma Chemical Company). Albumin was eliminated by centrifugating this suspension of mitochondria at 9,500 rpm for 10 min. The protein content in the mitochondrial fraction was determined by Lowry's method [49].

*2.6. Fluidity Changes of Mitochondrial Membranes.* 1,3 dipyrenylpropane (DPP) incorporation into membranes to form intramolecular excimers depends mainly on medium microviscosity and temperature of determination (24). Membrane fluidity is determined by estimating the excimer to monomer fluorescence intensity ratio (Ie/Im) of this fluorescent probe, a quotient that reflects lateral mobility of membrane phospholipids (25). Briefly, mitochondria were resuspended in Tris-HCl buffer (50 mM, pH 8) and then fragmented by sonication for 15 seconds before being separated by centrifugation at 13,000 rpm. The mitochondrial membrane pellet was resuspended and proteins were measured by Lowry's method. 0.1 mg of mitochondrial protein was mixed in a spectrofluorometric cell containing Tris-HCl (20 mM, pH 7.5). DPP solution in ethanol of spectroscopic grade was diluted (0.02 mg/mL) and mixed with membranes given a molar ratio of fluorescent probe to membrane phospholipids of 1 : 1400; these mixtures were incubated in darkness for 4 hours at room temperature. Fluorescence of DPP incorporated into membranes was measured at 24°C on a Perkin Elmer fluorescence spectrometer, LS50B. The fluorophore was excited at 329 nm and the monomer and excimer fluorescence intensities were read at 379 and 480 nm, respectively.

*2.7. Chromatographic Analysis of Fatty Acids.* Fatty acids from membranes were extracted with chloroform:methanol (2 : 1 vol/vol) and analyzed by gas-liquid chromatography. Briefly, C17:0 heptadecanoic acid, as internal standard, was added to 1 mg of mitochondrial protein and the mixture of methanol and chloroform, both dissolved in BHT, was added. After centrifugation, the chloroform phase was extracted and a second extraction was done by adding anhydrous sodium sulphate. The extract was evaporated under nitrogen, reacted with a mix of methanol, sulfuric acid, and toluene at 90°C for 2 hours, and then redissolved in hexane and a 5% saline solution. Following the extraction of the organic phase, the hexane was evaporated under nitrogen to obtain derivatized fatty acids to be placed into the injector of a Carlo Erba gas chromatograph with flame ionization detection; the temperature of the injector was 250°C and the oven temperature was maintained at 196°C, using helium as a carrier gas at 1.4 kg/cm$^2$.

*2.8. Chromatographic Anaylsis of Phospholipids.* Phospholipids were extracted with a methanol/chloroform solution mixed in a 2 : 1 ratio, dried in a SpeedVac, and then redissolved in chloroform. Following a second extraction adding anhydrous sodium sulphate, the solution was filtrated and then evaporated. Samples were analyzed by high-pressure liquid chromatography. Results are provided in relative percentage from the correspondent areas in the chromatogram.

*2.9. Chromatographic Anaylsis of Cholesterol.* Membrane lipids were extracted with chloroform:methanol (2 : 1 vol/vol) and analyzed by gas-liquid chromatography. Briefly, 10 $\mu$g of stigmasterol, as internal standard, was added to 1 mg of mitochondrial protein and the mixture of methanol and chloroform, both dissolved in BHT, was added. Extracted lipids react for 1 hour at 60°C with a mix of hexamethyldisilazane, trimethyl fluorosilane, and dry pyridine to convert free cholesterol and stigmasterol in their corresponding trimethyl esters. The mixture was evaporated with nitrogen and then redissolved in hexane to be injected into the Carlo Erba gas chromatograph with flame ionization detection; the temperature of the injector was set to 275°C, the temperature of the detector was 260°C, and that of the oven

was maintained at 275°C. Helium was used as a carrier gas at 1.5 kg/cm$^2$.

*2.10. Statistical Analysis.* All data are shown as means ± SE of triplicate experiments. Statistical analysis of the data for multiple comparisons was performed by two-way ANOVA followed by Student's tests. For a single comparison, the significance of any differences between means was determined by unpaired $t$-tests. The criterion for significance was $P < 0.05$ in all statistical evaluations.

## 3. Results

*3.1. Aβ at the Brain Enters the Neurons and Eventually Presents in Mitochondrial Membranes.* 12, 24, 36 and 48 hours following the intracerebral injection of fibrillar Aβ, deposits of Aβ forming aggregates were reactive to Aβ polyclonal antibody and revealed by immunohistochemistry. Congophilic amyloid deposits remained visible up to 21 days following the intracerebral injection (data not shown). Tissue sections of 50 μm, obtained with a vibratome, were used for immunoelectron microscopy and the Aβ positive immunoreactions were observed in mitochondria along the membranes and deep in the cristae. The presence of Aβ deposits in mitochondria was accompanied by a significant lost of their architecture, characterized by swelling, broken cristae, lost of membrane integrity, and vacuole formation (Figure 1).

*3.2. The Lost of the Cytoarchitecture Was Related to Free Radical Overproduction.* CM-H2XROS is a reduced, non fluorescent X-rosamine derivative, which is sequestered by mitochondria where it is retained and oxidized. Under oxidation, CM-H2XROS emits fluorescence as a consequence of the number of free radicals produced by mitochondria. This reagent is normally used in *in vitro* experiments after adding it to cells in culture. To demonstrate the effects of Aβ *in vivo,* we have introduced a variant by injecting CM-H2XROS intraperitoneally 15 minutes before tissue collections (as explained). Once the tissue was obtained, sections of the lesioned area were immediately cut in a vibratome and stained with Mito Tracker Green (MitGreen), which is essentially nonfluorescent in aqueous solutions, only becoming fluorescent once it accumulates within the lipid environment of the mitochondrion. Thus, the CM-H2XROS/MitGreen IOD quotient identifies the quantity of free radicals by the mitochondria present on each field of the microscope. We found a significant overproduction of free radicals both in Aβ- and in $H_2O_2$-treated brains ($P < 0.05$) as compared to PBS-injected brains. Brains of animals who had received melatonin showed significantly lower levels of free radicals ($P < 0.05$) (Figure 2).

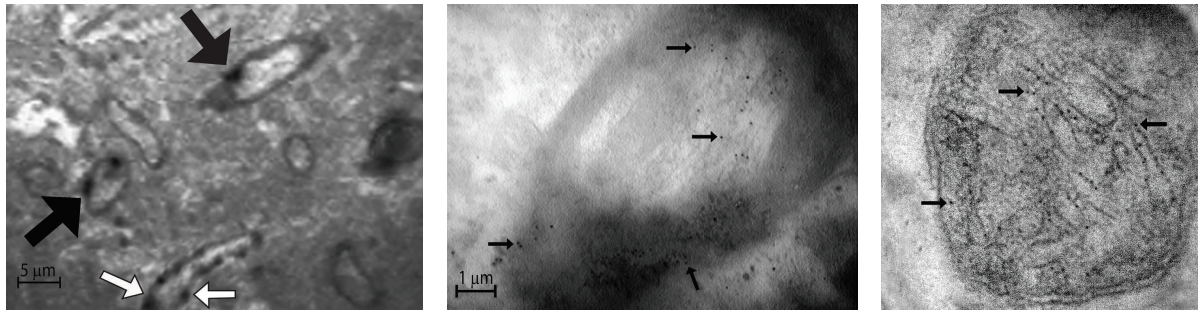
*3.3. Membrane Fluidity Was Inversely Correlated to Free Radical Overproduction.* The highest value in membrane fluidity was observed in PBS-injected brains, which showed the lowest amount of free radicals as well (Figure 3). The highest overproduction of free radicals, according to the CM-H2XROS/MitGreen IOD quotient, was observed in $H_2O_2$-injected brains. Interestingly, even though the production of free radicals in brains injected with fAβ was significantly higher than the quantity of free radicals observed in the PBS group, the difference between membrane fluidity and free radical overproduction was less obvious, as compared with the positive control group of $H_2O_2$. Brains of animals treated with melatonin had significantly reduced levels of free radicals and the difference between these and membrane fluidity was again obvious (Figure 3).

*3.4. The Unsaturated/Saturated Ratio, Significantly Affected by Aβ, Is Restored by Melatonin.* Aβ increased palmitic (16 : 0) and estearic (18 : 0) saturated fatty acids, 39 and 37% ($P < 0.05$), correspondingly. Additionally, Aβ reduced linoleic acid (18 : 2) at less than 35% the observed value in the PBS-injected brains ($P < 0.05$) and decreased linolenic acid (18 : 3) value 80% below the observed value in the PBS-injected brains. Additionally, Aβ significantly increased the polyunsaturated arachidonic acid (20 : 4) ($P < 0.05$, from 29.4 ± 2 to 51 ± 2.5). Thus, the elevated increase in saturated plus the severe decrement in linoleic and linolenic acids (Figure 4) was mostly responsible for an alteration in the ratio of unsaturated to saturated fatty acids in membrane phospholipids which is critical to normal cellular function.

The lower unsaturated to saturated ratio was observed in fAβ-injected brains to be even lower than the positive control group of $H_2O_2$, fAβ and $H_2O_2$ being the groups of study where the overproduction of free radicals was significant (Figure 2). With the use of melatonin, the U/S ratio returned significantly closer to control values (Figure 5), which reflected melatonin's role on each particular fatty acid (Figure 4).

However, the aforementioned showed that a drastic reduction in linolenic acid, a precursor of arachidonic acid (20 : 4, n6), and in linoleic acid, a precursor of docosohexanoic acid (22 : 6, n3), was reflected in the Aβ-induced levels of AA and DHA levels (Figure 6). In the Aβ-injected brains, arachidonic acid rose 20% in relation to the PBS-injected control values ($P < 0.05$), while DHA levels showed approximately 30% increase value ($P < 0.05$). This similar increment in both variables allows that the relationship between these relative values or DHA/AA ratio remains stable in Aβ-injected brains as compared to PBS-injected brains (Figure 6).

Except for the palmitic acid and the arachidonic acid, the rest of the Aβ-altered fatty acids tend to return to their basal levels, similar to PBS levels, when the animals were treated with melatonin (fAβ+Mel group), as shown in Figures 4–6. Another exception was the N3, 20 : 5, polyunsaturated eicosapentaenoic fatty acid (EPA) which seemed to be unaffected by Aβ (Figure 7). However, the EPA-to-AA ratio showed a significant reduction ($P < 0.05$). Interestingly, in Aβ-injected brains from melatonin-treated animals, EPA values were 60% and 43% higher than those observed in PBS- and in Aβ-injected brains. As a result, the EPA to AA ratio was restored (Figure 7).




(a) (b) (c)

Figure 1: Aβ stain by immunoelectron microscopy. 36 hours after the intracerebral injection of Aβ tissues from the injected area were obtained and subjected to immunohistochemistry by using a primary polyclonal antibody against Aβ. Deposits of Aβ forming deposits in the extracellular space were revealed by conventional light microscopy (data not shown). Aβ immunoreactivity was then revealed with a 6 nm gold label and observed in a transmission electron microscope which allows us to identify (a) deposits of Aβ within myelin axons (black arrows) and in the vasculature (white arrows). (b) Deposits of Aβ (black arrows) penetrate the axon membranes causing demyelination and appear in the axons. Axons look like bulb onions. (c) Aβ appears within the mitochondria finally, where it forms deposits along the cristae (black arrows) and causes intense inflammation, destruction of membranes, and vacuolization (magnification at 27800x).

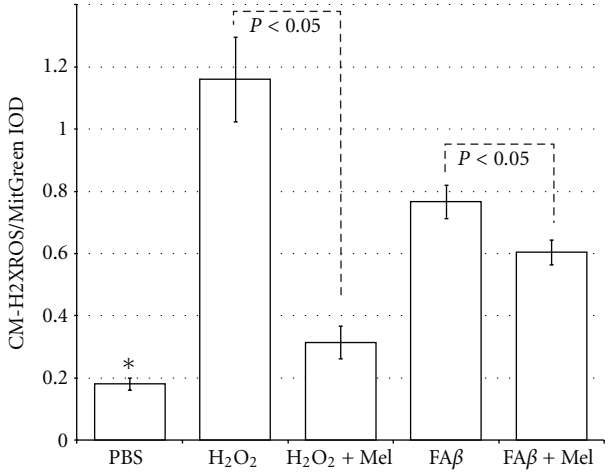



Figure 2: Compared with PBS-injected brains, those brains injected with Aβ or with $H_2O_2$ had a significant increase in free radical levels in mitochondria, according to the CM-H2XROS/MitGreen quotient (*$P < 0.05$ versus all the other groups). However, by using melatonin a significant decrease in mitochondrial free radicals was observed both in Aβ- and in $H_2O_2$-injected brains.

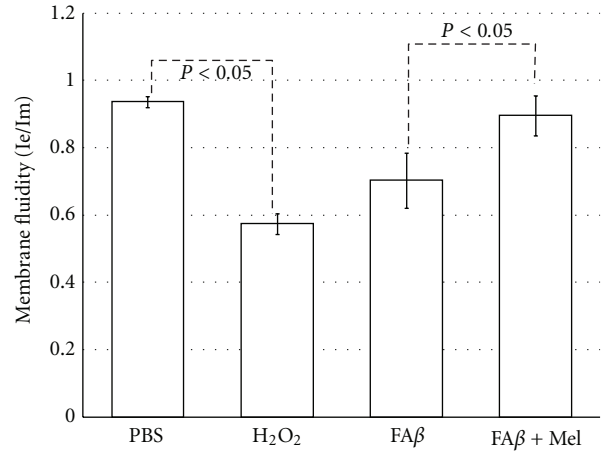



Figure 3: Brains of animals injected with Aβ showed a significant reduction in membrane fluidity as compared with PBS-injected brains, although less obvious than the observed in $H_2O_2$-injected brains, used as a positive control, which is in concordance with the degree of the free radicals overproduction, as shown in the previous graphic. Membrane fluidity in animals receiving melatonin was restored at the same level than the PBS group.

*3.5. Aβ Induced Significant Alterations in Mitochondrial Membrane Phospholipids.* Thus, while the effect of phosphatidyl ethanolamine (PtdEA) levels was reduced by a third in the fAβ group ($P < 0.05$), levels of phosphatidyl choline (PtdCHOL) were increased 40% ($P < 0.05$), but the phosphatidyl serine (PtdSER) values reached 120% as compared to PBS-injected brains (Figure 8). On the contrary, brains of animals treated with melatonin showed PtdEA levels similar to PBS-injected brains, while levels of PtdSER were significantly reduced with melatonin even beyond the control values ($P < 0.05$). PtdCHOL levels were also significantly reduced in presence of melatonin (Figure 8).

*3.6. Variations in Cholesterol Content Follow the Same Pattern As Variations in Free Radicals.* The lowest cholesterol values were found in the PBS-injected brains and the highest values were observed in the $H_2O_2$-injected brains ($P < 0.05$). The concentration of cholesterol in fAβ-injected brains was significantly higher ($59.6 \pm 5.7$ versus $47.6 \pm 5.2\,\mu$g/mg of protein, $P < 0.05$) than that in the control brains treated with PBS; this value was only 66% the value in $H_2O_2$-injected brains ($59.6 \pm 5.7$ versus $90.24 \pm 11$, data not shown). In spite of the significant increase in the membrane fluidity observed in fAβ+Mel brains, this change was apparently not related to the cholesterol content since melatonin did not change the levels of cholesterol in fAβ-injected brains (fAβ $60 \pm 6$ versus

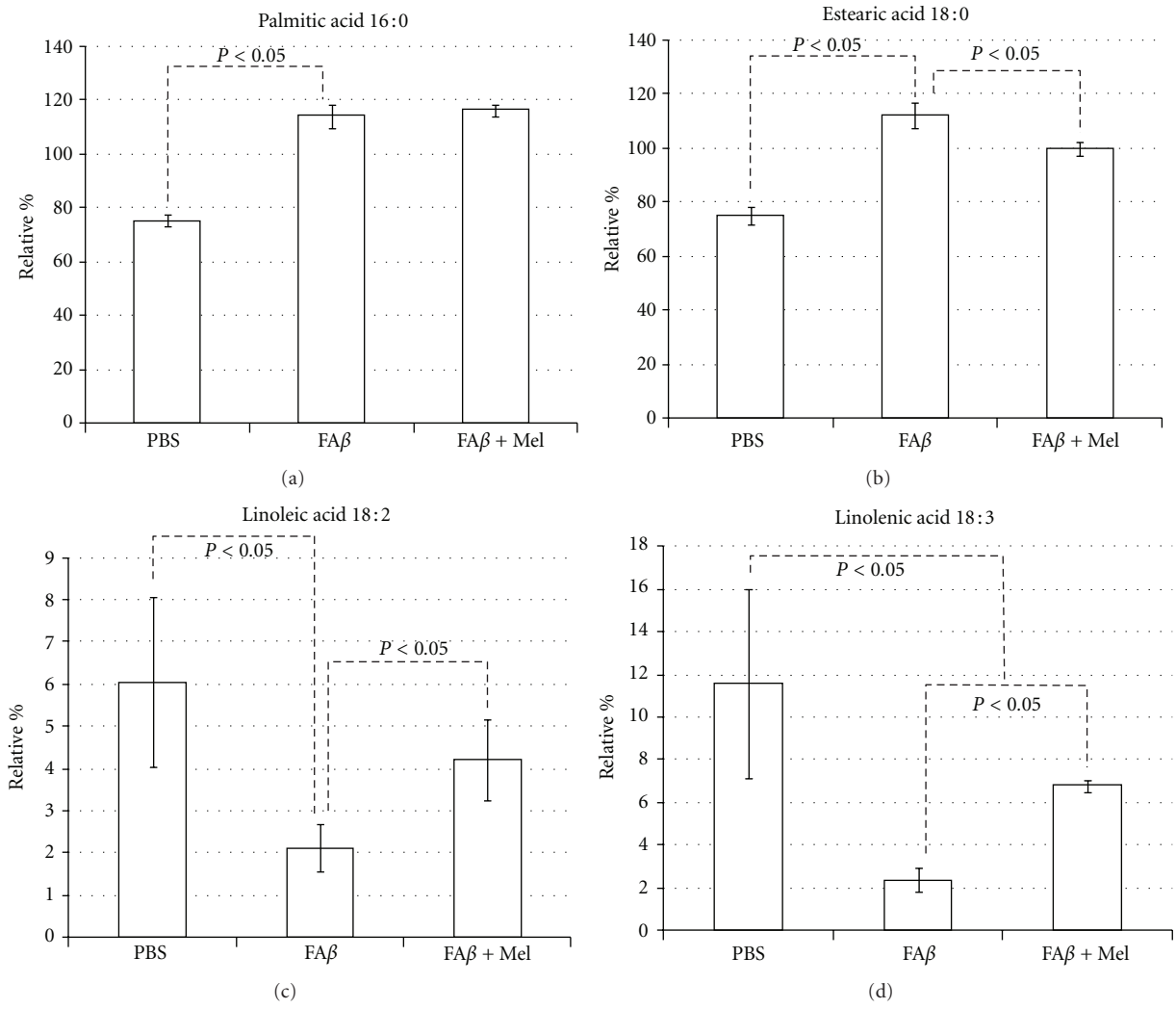



FIGURE 4: Aβ and $H_2O_2$ (not shown) had similar and highly significant effects on saturated fatty acids particularly on palmitic and estearic acids whose percentages were increased 39 and 37% correspondingly. Linoleic acid was reduced to a third from the control, while linolenic acid was reduced to less than a quart from the control value, as shown. These important effects of Aβ on specific saturated and unsaturated fatty acids affected the unsaturated/saturated (U/S) balance.

fAβ+Mel 61 ± 7) (Figure 9). The cholesterol-to-phospholipid ratio, on the other side, which reflects a loss of phospholipid and membrane rigidity, was significantly altered by Aβ, with the inability of melatonin to return it to the level found in PBS-injected brains (Figure 9).

## 4. Discussion

Lipid and fatty acid changes have been studied in plasma membranes, especially in both postmortem AD brain and transgenic mice. These changes have been associated with aging, Aβ deposits, dementia, and even mild cognitive impairment. Other experiments have been carried out *in vitro* by using purified synaptosomal plasma or mitochondrial membranes. To our knowledge, no studies have focused on mitochondrial membranes in response to *in vivo* extracellular deposits of fibrillar Aβ. In spite of its known ability to induce oxidative stress, alterations in lipid content of mitochondrial membranes induced by extracellular Aβ differed from those induced by $H_2O_2$.

Mitochondrial dysfunction has been related to oxidative stress in neurodegeneration as both cause and effect. At the same time, there is increasing evidence for membrane lipid, fatty acid, and cholesterol interactions with Aβ. These interactions have significant consequences in the pathogenesis of Alzheimer's disease. Our original aim was to demonstrate that extracellular deposits of Aβ, *in vivo,* would be able to induce mitochondrial failure as well as changes in mitochondrial lipid composition as a consequence of its ability to induce oxidative stress. $H_2O_2$ was chosen as positive control since endogenous hydrogen peroxide has been indicated as a secondary messenger mediating the intracellular effects of Aβ extracellular deposits. Furthermore, a relationship between

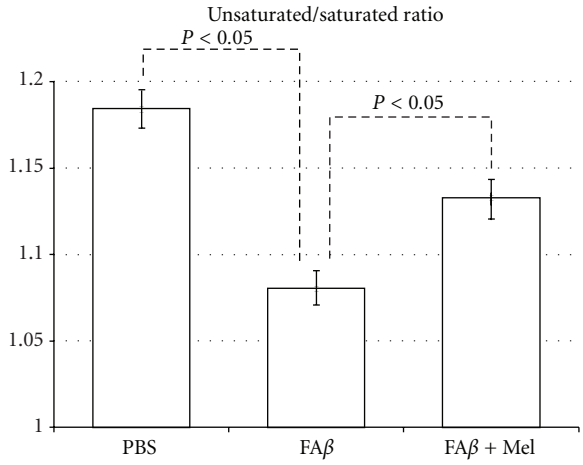



Figure 5: fA$\beta$-injected brains decreased significantly the U/S ratio, as compared with the PBS-injected brains. However, brains of animals taking oral melatonin showed a U/S ratio closer to the control group.

the accumulation of A$\beta$ monomers and oligomers and $H_2O_2$ production in the mitochondria of Tg2576 mice has been reported. However, we found that important A$\beta$-induced alterations were significantly different from those induced by $H_2O_2$. AA levels, for example, were significantly higher in the fA$\beta$ group than those in the $H_2O_2$ group ($P < 0.05$). The ratio U/S was not affected by $H_2O_2$, basically due to minimal effects on or to compensations between decrements and increments in one or another group of saturated versus unsaturated fatty acids. A$\beta$, increasing saturated fatty acids and decreasing unsaturated fatty acids, caused a persistent and significant decrement of the U/S ratio (Figure 5). This finding agrees with another work where the ability of A$\beta$ to interfere with the $\Delta$-9 desaturase enzyme was observed. $\Delta$-9 desaturase introduces the first double bond between carbon 9 and 10 of palmitoyl (16 : 0) or stearyl (18 : 0) Co A to form palmitoleic (16 : 1) or oleic (18 : 1) acids [19].

This profound fatty acid imbalance was reflected in phospholipid levels, in such a manner that $H_2O_2$ did not affect the levels of PtdEA, but A$\beta$ reduced significantly this phospholipid. However, the most severe effect of A$\beta$ was observed in PtdSER, causing a 5-fold increment, whereas $H_2O_2$ did not affect this parameter.

There seems to be a different mechanism of damage. While the strict oxidative damage, represented by our positive control group $H_2O_2$, caused the highest increment in PtdEA levels, A$\beta$ reduced PtdEA levels and, on the contrary, increased very significantly the PtdSER levels and, less important but also significantly, the PtdCHOL levels (Figure 10).

Thus, A$\beta$ deleterious effects were not oxidative stress related—or at least not completely explained by oxidative alterations—which is evident when comparing the differing effects of $H_2O_2$ and A$\beta$ on membrane lipids ($P < 0.05$). Fatty acid (FA) composition of phospholipids determines biophysical (and functional) characteristics of membranes (e.g., membrane fluidity) and plays an important role in cellular integrity and intra- and intercellular communication. We found a significant ($P < 0.001$) inverse correlation ($r^2 = -0.74$) between mitochondrial membrane fluidity and cholesterol content. Indeed, we found that A$\beta$ and $H_2O_2$ caused the more severe oxidative stress, the lowest membrane fluidity, and the highest cholesterol content. However, in the A$\beta$ group the reduction of oxidative stress seemed not to affect the cholesterol increment, although A$\beta$ had a more severe effect on fatty acids (Figures 5, 6, and 7) and phospholipid redistribution (Figure 10).

A tendency of cholesterol to aggregate into clusters at a cholesterol/phospholipid ratio of greater than 0.3 is known since 1972 [50], and we have found a $0.3 \pm .08$ cholesterol to phospholipid ratio in fA$\beta$-injected brains due principally to a severe decrement in PtdEA against an increment in cholesterol content (Figures 8 and 9). Cholesterol aggregates in membranes are a well-known characteristic of A$\beta$-induced damage [30, 51, 52]. High-cholesterol diet has been associated with increased deposits of A$\beta$ [53], and we have found [54] that animals fed with a cholesterol-enriched diet presented a significant increase in mitochondrial structural damage linked to severe dysfunction of this organelle. It was noticeable that, according to our results, melatonin could not impair this cholesterol re-arrangement but was able to induce a significant increment in membrane fluidity (Figure 3). This phenomenon illustrates the role of lipid peroxidation and the reaccommodation of phospolipids, particularly PtdSer and PtdEA, along the membranes as determinants of membrane fluidity, beyond the role of cholesterol [55].

A$\beta_{42}$ oligomers accumulate more slowly and in reduced amount at the plasma membranes of fibroblasts from familial AD (FAD) patients enriched in cholesterol [56]. On the other side, it is also reported that A$\beta$ binds lipids, but with a higher affinity for cholesterol than PtdCHOL or saturated fatty acids [51]. We may therefore speculate, according to our results, that the cholesterol rearrangement observed in brain cells may be a defensive response against oxidative stress, with a secondary effect, A$\beta$ binding.

Specific alterations in fatty acids have been related to A$\beta$ pathogenesis. For example, unsaturated fatty acids oleic acid, and linoleic acid, have been shown to increase the $\gamma$-secretase activity and A$\beta$ levels, as evaluated in PSwt-1 cells, which contains the wild-type human presenilin 1 (PS1) and wild-type human APP full-length cDNAs, [11]. According to our results, A$\beta$ decreased severely the content of linoleic acid (6.5 mol% in PBS-injected brains versus 2.12 mol% in A$\beta$-injected brains, $P < 0.05$), as observed in old Wistar rat brain, which implies that this effect occurs regardless of the ApoE phenotype.

The importance of the ApoE phenotype involves the carrying of proteins in combination with lipids to form lipoprotein particles with hydrophobic lipids at the core and hydrophilic side chains made of amino acids. ApoE also aids the transport of triglyceride, phospholipid, and cholesterol into cells, by mediating the binding, internalization, and catabolism of lipoprotein particles [57]. ApoE is considered a risk factor in AD because 40–65% of AD patients have at least one copy of the 4 alleles; although the exact mechanism

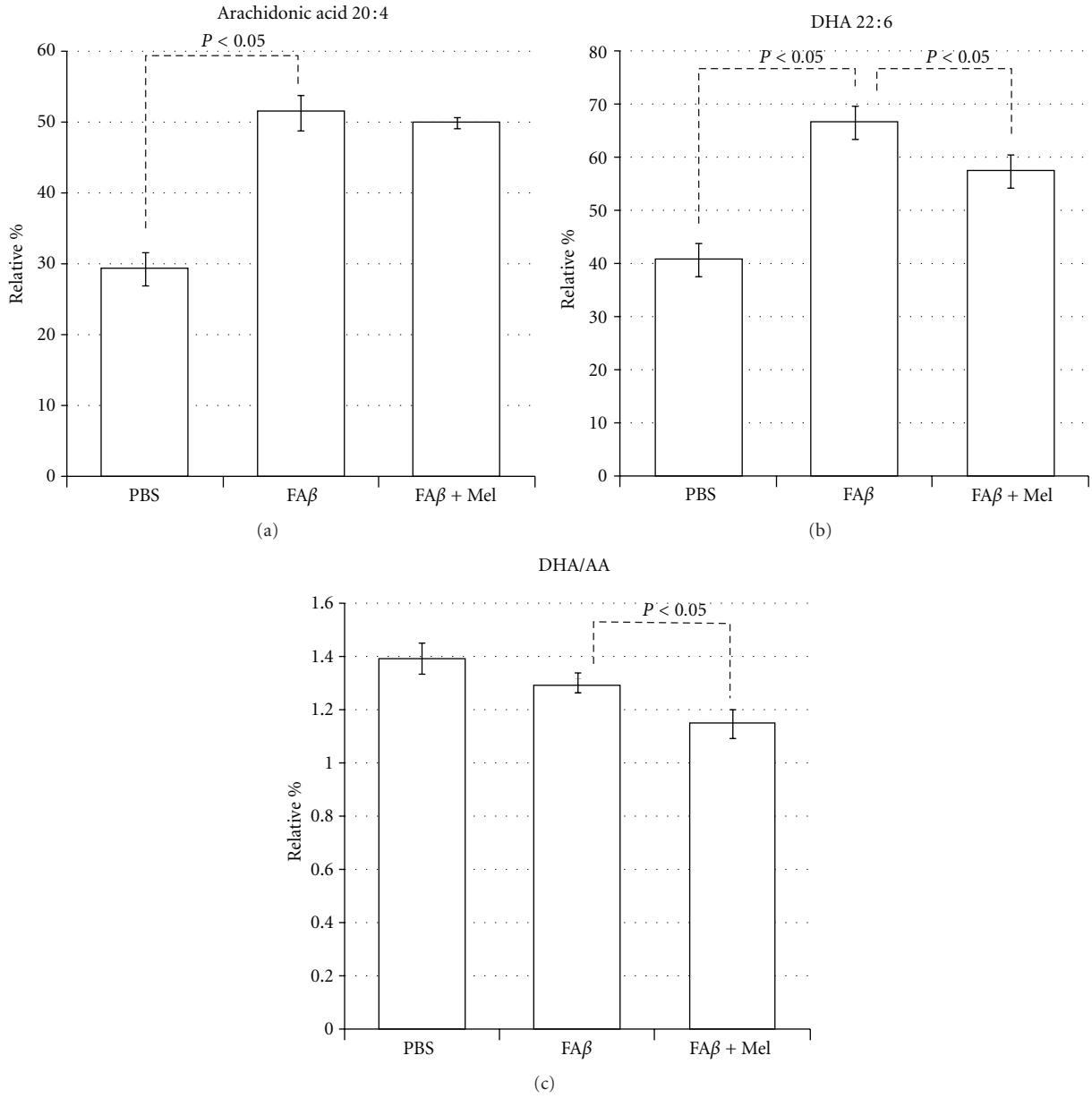



Figure 6: A$\beta$ and $H_2O_2$ (not shown) produced important increases in both n6 and n3 PUFA, which reflects the previous described changes in free fatty acids. A similar increase in DHA and AA allowed the DHA/AA ratio to remain stable, when compared with the PBS group. It is obvious that melatonin reduces the DHA/AA ratio, particularly at the expense of a decrease in DHA levels.

of this feature remains to be fully determined, an interaction with amyloid insoluble protein aggregates or with APP seems to be involved [58, 59]. How ApoE controls brain lipids and how this regulation may impact the clearance of A$\beta$ or the progression of damage are less clear [60]. In postmortem brain samples, no significant difference in lipids or fatty acids was found between AD patients classified as homozygous for ApoE4 and those classified as heterozygous or having no ApoE4 [10, 61]. Thus, ApoE genotype on fatty acids and lipid composition and/or its distribution in brain cell membranes seem to have no significance and would not bias our results. Additionally, by comparing the association of human, rat, and rabbit ApoE with A$\beta$, a similar lack of affinity for A$\beta$ between rat ApoE and human ApoE4 has been reported [62]. Thus, rat ApoE, the same as the AD-related ApoE4, does not form complex with A$\beta$.

Another fatty acid whose relationship with A$\beta$ pathogeny has been widely studied is AA. This N6 PUFA is an agonist of proinflammatory pathways, which additionally as has been reported increase the levels of A$\beta$ [63]. It is known also that A$\beta$ oligomers trigger neuronal apoptosis by early activation of a cPLA2-dependent pathway leading to production of AA [14]. We found that the AA precursor linoleic acid was reduced while AA was increased by A$\beta$, which supports the

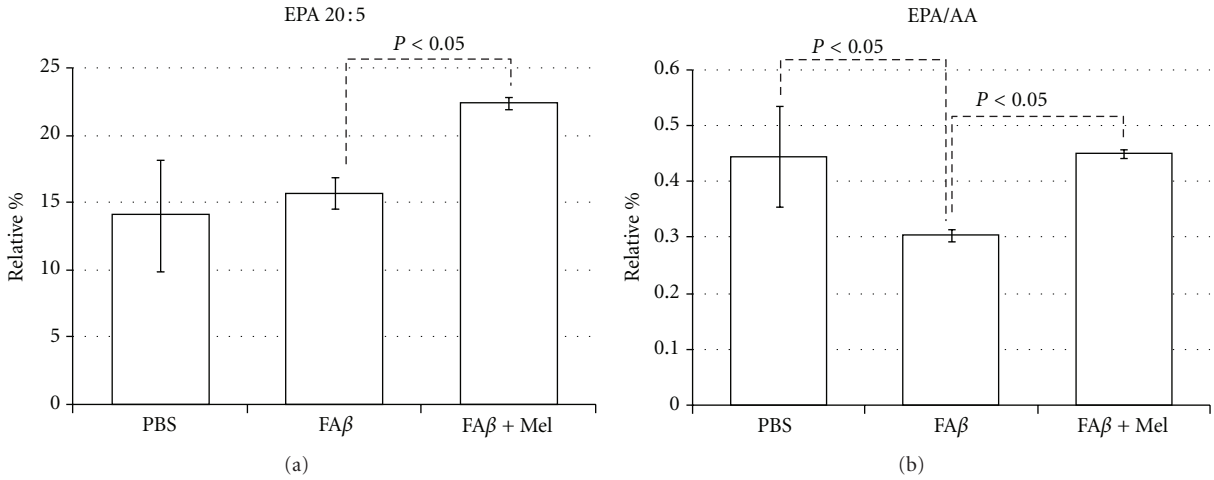



Figure 7: EPA was not to significantly responsive to A$\beta$. However, in the presence of melatonin and contrary to the results with the other major n3 PUFA, DHA, the relative percentage of EPA rose significantly, which impacted the EPA/AA ratio, as shown.

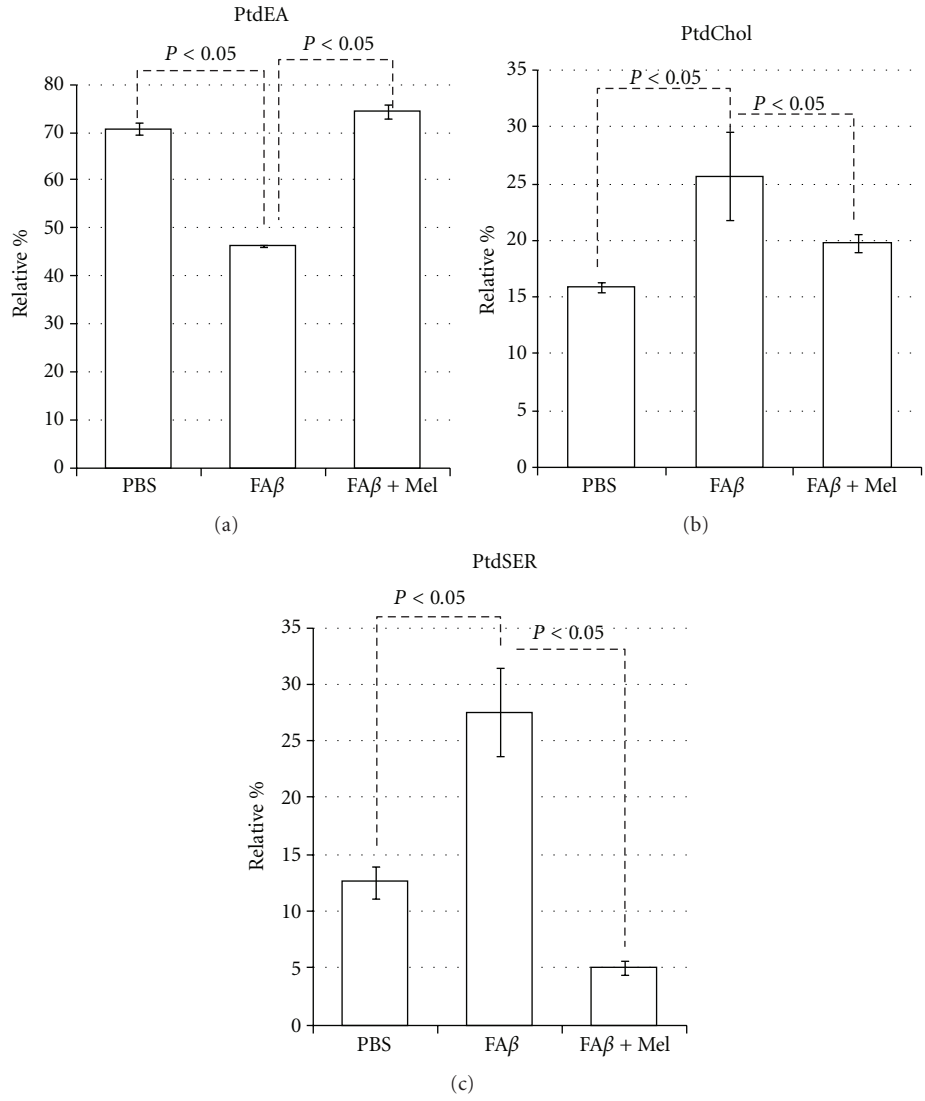



Figure 8: A$\beta$ decreased significantly the PtdEA levels and increased the levels of PtdCHOL and PtdSER, the latter with a 5-fold increment. Results are expressed in relative percentage ± standard error.

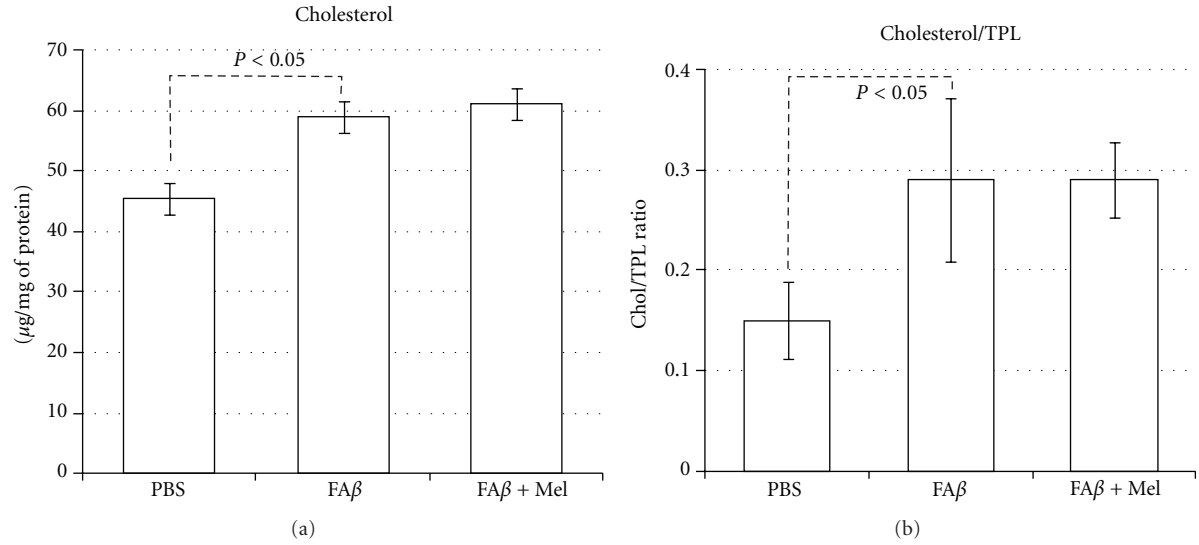



Figure 9: Cholesterol content in mitochondrial membranes is significantly increased in fA$\beta$ injected brains. The $H_2O_2$ control group (data not shown) and the fA$\beta$ experimental group, which showed the more important overproduction of free radicals and the lowest membrane fluidity, coincide with the highest cholesterol content. However, in spite of its ability to scavenge free radicals and restore membrane fluidity, melatonin was unable to reduce cholesterol content in mitochondrial membrane. Compared according to their relative values, cholesterol and total phospholipids ratio was significantly altered.

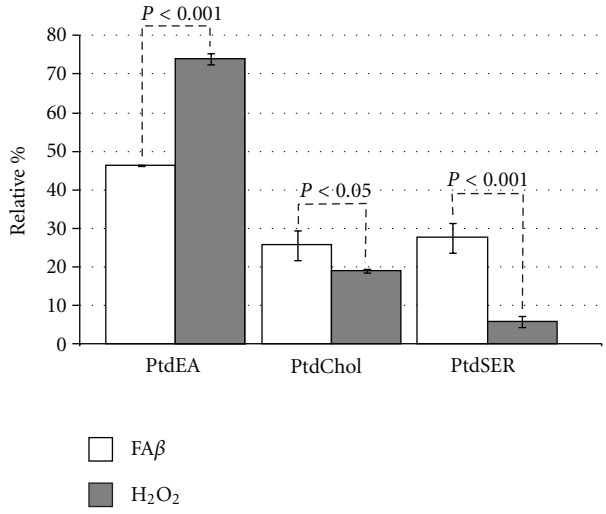



Figure 10: Significant differences between A$\beta$-injected brains and $H_2O_2$-injected brains.

proposal that A$\beta$ paves its own way by changing the quality and distribution of lipid membranes.

Herein we report important alterations in mitochondrial membranes following the intracerebral injection of A$\beta$. There is important in this context the relationship between n6 and n3 PUFA, particularly the relationship between the proinflammatory AA with its counterparts DHA and EPA. EPA and DHA differ in their effects on plasma lipid profiles, gene expression, and neural membrane structure. EPA downregulates the enzymes involved in DHA synthesis and decreases DHA synthesis from its precursor, $\alpha$-linolenic acid [64]. We have found that A$\beta$ increased both DHA and AA, while the levels of EPA remained stable, but the treatment with melatonin, which did not affect the levels of AA, was able to increase very importantly EPA. EPA has been reported as anti-inflammatory upon several conditions and different cell types, but importantly in aging and A$\beta$-induced neuroinflammation [65–67]. Specifically EPA is linked to a modulatory role in microglial activity [68]. We have reported a remarkable reduction in microglial activity in rats intracerebrally injected with A$\beta$ but under melatonin treatment [4].

Even though being examined in a different context, there is a report where melatonin was found protective of AA, DHA, and EPA. Arachidonic acid was protected more efficiently than DHA and EPA at all the melatonin concentrations examined when rat liver microsomes were incubated with ascorbic acid [69], which is quite similar to our results (Figures 6 and 7). This phenomenon is linked to protection against lipid peroxidation, a remarkable ability of melatonin supported by its amphoteric nature as well as its ability to cross the blood-brain barrier (BBB) and enter into the central nervous system [70].

Evaluated in cortical synaptosomes from gerbils, a loss of phospholipid asymmetry induced by $A\beta_{1-42}$ has been reported [71]. This phenomenon implies the oxidative modification of the flippase enzyme by reactive alkenals which causes externalization of PtdSER and the subsequent phospholipid asymmetry, which in turn causes membrane dysfunction, $Ca^{++}$ massive influx, and apoptosis. The anionic PtdSER also may increase the fibrillization of A$\beta$ [72]. We found that A$\beta$ causes a 120% increase in PtdSER levels. However PtdSER levels in brains of animals which received melatonin treatment decreased 5 times compared to brains from animals without melatonin treatment.

## 5. Conclusions

The relationship between membrane lipids with A$\beta$ is usually focused on how lipids may allow, facilitate, or even induce the amyloidogenic processing of APP. This relationship is also explored to explain how A$\beta$ causes cellular dysfunction.

Our approach to the *in vivo* study of the A$\beta_{1-42}$ peptide, the predominantly neurotoxic form of A$\beta$, was to inject the peptide directly into the hippocampus and then examine the relationship with membrane lipids, in order to explain how A$\beta$ may penetrate the cell and then approach to mitochondria and cause the well-known severe dysfunction of this organelle.

The intracellular amyloid cascade is, of course, widely studied and elegantly explained [73–75]. It is also likely that the pathogenically critical process of A$\beta$ oligomerization may begin intraneuronally and the energy hypometabolism may appear before the presence of senile plaques or neurofibrillary tangles [76]. However, without discarding the previous statements, there is evidence to consider the extracellular A$\beta$ as the principal source of intracellular A$\beta$, given the huge amounts of A$\beta$ in aggregates, the physical properties of this peptide, and its ability to alter fatty acids and lipids on membranes, either because of its pro-oxidant activity or because of its physical interactions with lipids.

We reported how exogenous A$\beta$ forms deposits in the extracellular space, then presents inside the cells—particularly through the axons causing demyelination, which agrees with other reports [5, 6]—and, finally, how A$\beta$ is found inside mitochondria where it causes severe structural damage linked to free radical overproduction and significant alterations in mitochondrial membrane lipids.

By using melatonin, it is possible to ameliorate the membrane fluidity without affecting cholesterol content in membranes, while it restores the balance of lipids. Importantly, melatonin reduces the negatively charged PtdSER in membranes and, by this means, might impair the toxicity of A$\beta$. Another important feature is how melatonin may increase EPA content in membranes, restoring the EPA/AA ratio, a phenomenon widely known by its anti-inflammatory effects. Melatonin restores membrane structure and functionality, an effect which exclusively could not be attributed to its antioxidant capacity.

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*Review Article*

# Alternative Strategy for Alzheimer's Disease: Stress Response Triggers

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Stress resistance capacity is a hallmark of longevity protection and survival throughout the plant and animal kingdoms. Latent pathway activation of protective cascades, triggered by environmental challenges to tolerate heat, oxygen deprivation, reactive oxygen species (ROS), diet restriction, and exercise provides tolerance to these stresses. Age-related changes and disease vulnerability mark an increase in damage, like damage induced by environmental challenges. An alternative approach to immunotherapy intervention in Alzheimer's Disease is the use of mimetics of stress to upregulate endogenous protective cascades to repair age damage, shift the balance of apoptosis to regeneration to promote delay of onset, and even progression of Alzheimer's disease memory dysfunction. Mimetics of environmental stress, hormetic agents, and triggers, endogenous or engineered, can "trick" activation of expression patterns of repair and rejuvenation. Examples of known candidate triggers of heat response, endogenous antioxidants, DNA repair, exercise, hibernation, and telomeres are available for AD intervention trials. Telomeres and telomerase emerge as major regulators in crossroads of senescence, cancer, and rejuvenation responsive to mimetics of telomeres. Lessons emerge from transgenic rodent models, the long-lived mole rat, clinical studies, and conserved innate pathways of stress resistance. Cross-reaction of benefits of different triggers promises intervention into seemingly otherwise unrelated diseases.

## 1. Introduction

Divergent biological phenomena have fundamental convergent pathways that affect aging, age-related diseases, and stress resistance responses. Hormetic stress pathways are activated by environmental chemical and physical cues, that are beneficial at threshold low levels but are otherwise toxic agents at higher levels [1]. Nature preserves those organisms and small molecular triggers that promote tolerance responses to environmental stress including, youthful restoration of DNA repair, resistance to oxidizing agents, protein structure and function repair, improved immunity, tissue remodeling, and altered metabolism [2]. Survival pathways in ancient species exist in present species and when activated, show potential for increased longevity and latent rejuvenation potential regardless of divergence of the hormetic stressing agent. Environmental stress of UV and photoreaction, activates survival pathways to rejuvenate cells and increase lifespan in paramecia [3], and induces radiation resistance and DNA repair in human cells in culture [4]. Common key regulators and pathways respond to diverse challenges of physical and chemical stresses of temperature, diet, exercise, hibernation, and radiation. Both posttranslational and transcriptional activation of latent pathways responses involves epigenetic modifications by deacetylation, phosphorylation, methylation, ubiquitination, and mechanisms used in differentiation to provide stress resistance. As a consequence of common protective pathways, cross-resistance to pathologies that share common cellular cues represents an under-used strategy in disease intervention; that is, drugs effective in divergent diseases may show benefit in acute and chronic dysfunctions and have application in intervention in Alzheimer's disease.

The goal of intervention strategy reviewed here is to decrease vulnerability and rescue in Alzheimer's disease, by activation of stress resistance pathways. Triggers mimic environmental stresses including oligonucleotides, heat shock, exercise, and hibernation drugs, known to activate key

regulators of protective metabolic pathways to restore homeostasis, and proposed to provide resistance and repair of oxidative DNA and protein damage induced by AD.

This review focus is on lessons learned from the role of stress resistance triggers, hormesis, and telomeres, in rodent models of induced senescence, successful aging in the mole rat, and obstacles encountered in immunological therapy in clinical studies to provide a basis for intervention strategy for AD.

## 2. Mimetics of Stress Resistance

Stress resistance is key for survival and maintenance of the species, and nature has preserved survival pathways from single cells to man. Since the appropriate threshold of low-dose beneficial versus toxic dose of environmental and chemical stress is difficult to assess, the use of mimetic agents of these stresses offers better dosage control to avoid high-dose stress damage [2]. Mimetics can trigger stress-related transcriptomes, expression of families of genes activated by a common transcription factor, that provide benefit not only the targeted beneficial response, but also youthful rejuvenation, and improvement of multiple avenues to stress resistance to intervene in multiple age-related disease [5–7]. These fundamental survival pathways, lifespan assurance loci, master regulators, also called vitagenes, confer plasticity to species longevity, lifespan extension, rejuvenation, and repair [2, 5–12]. As the molecular roles of aging, stress and neurodegenerative disease are elucidated, oxidative stress emerges as a common damage denominator and activation of pathways used in early development; that is, FOXO and IGF-1, also serve roles in mitigation of stress resistance and disease [13–15].

## 3. Radiation Stress

Mimetics of UV damage include the use of DNA oligonucleotides homologous to the telomere (TTAGGG repeat, "T-oligo's") as triggers to activate innate telomere-based protective responses that act to reduce DNA and oxidative damage to cells [4]. The antioxidative pathways induction, by T-oligo's, makes these UV mimetics potential candidates for relief from induced oxidative toxicity in AD and cancer. More recently, telomere homolog oligonucleotides show induction of apoptosis in malignant, and not normal lymphoid cells, to provide potential anticancer therapy potential [16].

## 4. Protein Structure and Function Stress Damage

Protein misfolding and aggregation from single cells to multicellular organisms dramatically affect normal cell structure and function needed for survival [17] and is a hallmark of AD. The rescue of neuron protein damage involves activation of the heat shock response, and FOXO, and SIRT-1 to restore protein homeostasis [18, 19]. Protein homeostasis (proteostasis) is achieved by how high the threshold of the stress response is set to detect and combat protein misfolding. The heat shock factor 1 (HSF1) regulates the response to the metabolic state of the cell and centralized neuronal control that allows optimal resource allocation between cells and tissues. HSF1 activation requires a stress-activated NAD+-dependent SIRT1 deacetylase and phosphorylation, to signal transcription of molecular chaperones that resolve misfolded and aggregated proteins [19]. Misfolded proteins, whether a consequence of aging, toxins, hypoxic, oxidative, or ischemic stress, signal cell death damage, proapoptosis responses, that impact longevity, and disease states. HSP 70 heat shock protein is a major rescue response to damage that impacts longevity [20, 21], vulnerability, and progression of AD neuronal pathology. Hormetic agents are candidates to intervene in proteotoxic damage and associated clinical symptoms [22, 23] and are identified here.

Ethanol is a candidate hormetic trigger to induce the heat shock response [23] and thus has potential for intervention in AD. Ethanol preconditioning inhibits amyloid-Beta-induced neurotoxicity and apoptosis [24]. Constitutive and inducible HSP70s are involved in oxidative resistance evoked by heat shock and ethanol. In the brain, moderate ethanol pretreatment causes an almost 3-fold increase in brain levels of heat shock protein HSP 70 and can prevent beta-amyloid peptide (Abeta)-induced neurotoxicity and apoptosis in organotypic hippocampal-entorhinal slice cultures [24]. Neuronal protection by ethanol pretreatment reduces behavioral deficit, neuronal death, and delays neuronal death, neuronal and dendritic degeneration, oxidative DNA damage, and glial-cell activation after ischemia/reperfusion (I/R) challenge [25] and prevents postischemic leukocyte-endothelial cell-adhesive interactions [26].

Another trigger of protection against oxidative damage known to induce endogenous antioxidants and HSP70 is an acyclic isoprenoid. Geranylgeranylacetone (GGA) is a nontoxic HSP70 inducer of HSP70 with beneficial responses including reduction of inflammation in gastritis, apoptosis, induction of protective pathways like thioredoxin, and antiviral genes that offer a generalized upregulation of disease immunity [27, 28].

Activation of endogenous antioxidants is an alternative approach to upregulate natural defenses against oxidative damage and associated pathologies of neurodegenerative disease and including AD. Activators of the "Antioxidant Response Element" include oltipraz, and ferritins. Oltipaz is a substituted 1,2-dithiole-3-thione, originally developed as an antischistosomal agent, that possesses chemopreventive activity by transcriptional activation of a gene cascades involved in carcinogen detoxification and attenuation of oxidative stress [29]. Exposure of rodents to 1,2-dithiole-3-thiones trigger nuclear accumulation of the transcription factor Nrf2 and its enhanced binding to the "antioxidant response element" (ARE).

Ferritins, an ancient family of protein nanocages, also participate in activation of the ARE-responsive element. Ferritins concentrate iron in iron-oxy minerals for iron-protein biosynthesis and protection against oxy radical damage. The promoter of human ferritin-L contains an overlapping Maf recognition element (MARE) antioxidant

responsive element (ARE). Thoreductase can be transcriptionally activated by sulphorane and other electophiles by the antioxidant response element ARE. The ferritin receptor is activated by tert-butylhydroquinone, sulforaphane, and hemin with responses comparable to thioredoxin reductase, ARE regulator or quinone reductase (MARE/ARE regulator) [30].

## 5. Hibernation and AD Intervention

Hibernation is a classical beneficial response to environmental stresses of depleted energy stores, intracellular acidosis, hypoxia, hypothermia, cell volume shifts, and inactivity induced muscle wasting [31] characterized by epigenetic modulation affecting transcriptional and translational controls [32, 33]. Animals do not need to undergo a torpor state, to benefit from activation of at least some of the hibernation protective pathways. Use of Hibernation Induction Triggers, identified to activate protective hibernation gene cascades, especially using deltorphins opioid receptor agonists as mimetics of hibernation, shows reduction of damage in rodent model systems of heart attack [7], stroke, and hemorrhage shock [34–39]. The cardioprotective mechanism of deltorphin II is mediated via stimulation of peripheral delta (2) opioid receptors that involve protein kinase C, NO-synthase, KATP, and the autonomic nervous system to induce both its infarct-sparing and antiarrhythmic effects [37]. Neuroprotection by both hibernating woodcock serum and deltophin E was demonstrated in an neuronal ischemic stress rodent model [38]. The delta-2 opioid receptor agonist activation of protective pathways includes anti-inflammatory properties [39] that likely contribute to proven resistance to shock, that may also reduce AD pathology and progression.

Metabolic changes also characterize hibernation. Upregulation of fatty acid-binding proteins during hibernation facilitates the switch to a primary dependence on lipid fuels by nearly all organs. Changes in hibernation include upregulation of key regulators of energy metabolism and mitochondrial biogenesis, namely, PPAR gamma transcription factor and its coactivator, PGC-1. Several hypoxia-related genes including HIF-1alpha are also upregulated during hibernation suggesting a role for this transcription factor in mediating adaptive metabolic responses for hibernation [32, 33] useful in intervention potential for AD and diabetes.

AICAR, (Aminoimidazole-4-carboxamide-1-$\beta$-4-ribofuranoside) an agonist of AMPK, is a mimetic of exercise that upregulates pathways common to exercise including the key PGC-1 energy regulator [40]. In theory, and in experimental studies, AICAR intervenes in acute ischemic stress, by activation of protective pathways that are anti-inflammatory, anti-oxidative stress, and prosurvival pathways that promote intervention in ischemic stress pathways induced by exercise. Indeed, in our recent studies, AICAR pretreatment and posttreatment significantly increases tolerance and survival to a severe hemorrhage model of ischemic stress [41].

AICAR is a very promising candidate for pretreatment of early and late AD since evidence shows that AICAR treatment increased PGC-1alpha as a mimetic of stress. Increases PGC-1 levels dramatically protect neural cells in culture from oxidative-stressor-mediated death and making PGC-1 a target molecule for therapeutic manipulation oxidative stress [42] and candidate target molecule in AD therapy. AICAR intervenes in LPS/A beta-induced inflammatory processes by blocking the expression of proinflammatory cytokines, inhibits reactive oxygen species in astroglial cells, and promotes NGF-induced neurite growth in PC-12 cells [43]. PGC-1 is identified as a target molecule for diabetes as well [44].

Related studies use nutritional supplements to increase heat shock proteins and key metabolic regulators. Acetyl-L-carnitine induces upregulation of heat shock proteins and protects cortical neurons against amyloid-beta peptide 1–42 mediated toxicity and, thus, is nutritional candidate for intervention in AD [12, 45, 46]. Resveratrol, as well, is among the potential supplement s for AD via manipulation targeting activation of the Sirt-/PGC-1 neuroprotective axis [19, 47]. Other supplements and cocktails are recommended in other studies and reviews.

Mimetics of environmental stress, in the seemingly unrelated phenomena, hibernation, exercise, heat attack, stroke, severe hemorrhagic stroke trauma, metabolic diseases, and neurological disorders and AD, share common denominators, ischemic, metabolic, protein misfolding, and oxidative stress. The induction of protective pathways that promote survival instead of apoptosis and cell death, associated with energy deficits and inflammatory processes share drug benefits despite the disparity in the acute and chronic disease states. Diabetes drugs then may have potential for AD therapy.

## 6. Telomeres, Aging, and Alzheimer's Disease

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by the addition of the telomere repeat, TTAGGG, that declines with age. Telomerase and telomeres are the subject of thousands of current studies and reviews that link telomeres to aging and cancer. It is clear that telomerase function is not restricted only to repair of lost telomere length with age and may interact with the polycomb complex, that impacts various biological processes, including differentiation, maintenance of cell identity, cell proliferation, and stem-cell plasticity. Decline in telomere function links mitochondria, stem cells, and metabolic compromise [48, 49].

Genetically engineered telomerase-deficient mice are a model system that shows in vivo wide-spread endogenous DNA damage, tissue atrophy, stem-cell depletion, organ system failure and impaired tissue response that mimic age-related changes. The reversal of tissue degeneration in aged telomerase-deficient mice by genetically engineered inducible telomerase activation shows unprecedented evidence for global regeneration of organ systems [50]. Telomerase reactivation in late generation TERT-ER mice rejuvenates mice. The telomerase induction extends telomeres, reduces DNA damage, associated cellular checkpoint responses, restores proliferation in quiescent cultures,

eliminates degenerative phenotypes across multiple organs, reverses neurodegeneration, and restores the innate behavioral olfactory avoidance responses [50]. Evidence, that such regeneration occurs in normal aging with activation of telomerase, is, as yet, not available. The role of telomerase and cancer is still unraveling; mimics of UV irradiation, telomere oligonucleotides, induce apoptosis in malignant but not normal cells [16] and offer an anticancer potential for telomere damage response.

Longer telomere length usually correlates with positive survival response and stress resistance [51]. However telomere *shortening* reduces amyloid brain pathology in mice [52] and mole rats, a rodent model of successful aging, does not show age-related disease vulnerability, and has short telomeres [53].

The role of telomeres in aging and disease is of major importance as knowledge of operative mechanisms unravel in normal and disease states. At present, telomerase induction appears as an antiaging and rejuvenation potential that may delay vulnerability to AD; however, it is too premature to predict benefits and adverse side effects of treatment. Activation of stem cell repair is a projected pathway with advantage potential for AD.

## 7. Senescence versus Rejuvenation

The antithesis of telomerase promotion of cell replication and growth is the p16INK4 locus involved in promotion of senescence. The Ink4a/Arf locus encodes 2 tumor suppressor molecules, p16INK4a and Arf, considered the principal mediators of cellular senescence [54, 55]. Expression of p16INK4a and Arf markedly increases in almost all rodent tissues with advancing age, while there is little or no change in the expression of other related cell cycle inhibitors. The age-related increase in Ink4a/Arf expression can be independently attributed to the expression of Ets-1, a known p16INK4a transcriptional activator, as well as unknown Ink4a/Arf coregulatory molecules [54, 55]. Genetic data have firmly established that both p16INK4a and ARF proteins possess significant in vivo tumor suppressor activity. The anti-cancer growth inhibitory activity of the p16INK4a and ARF locus that can arrest cell growth benefit unfortunately can arrest cell growth in cells possessing self-renewal potential like tissue stem cells with a resulting decline the regenerative capabilities of the organ maintained by that stem cell. Decline of this stem cell reserve is a cardinal feature of mammalian aging marking the expression of the INK4a/ARF locus, not only to be a major suppressor of cancer, but also an effecter of mammalian aging [54]. Mimetics that tips the balance between INKA and telomerase, without cancer promotion, are candidates for successful aging. There is already evidence that the replicative state of the cell, normal or cancer, can determine response to telomerase induction [16]. The telomerase global potential is, at once, awesome and frightening; evidence is that aside from extension of telomeres, telomerase is a master regulator with potential for regulation of hundreds of genes with unknown immediate or long-term adverse side effects on nondividing brain cells in normal human aging. The telomere long length and telomerase rejuvenation potential, though highly correlated with longevity, can be independent, as found in the mole rat short telomeres with long life.

## 8. Successful Aging Model

In contrast with the multiple mouse models of disease and age-accelerated systems, the naked mole rat, living in burrows in arid and semiarid burrows in Africa, represents a model of successful aging. The mole rats are the longest-living rodents known, with a maximum lifespan of 30 years, at least 5 times or longer than expected on the basis of body size [53]. For at least 80% of their life, mole rats maintain normal activity, body composition, reproductive and physiological functions with no obvious age-related increases in morbidity or mortality rate, and cancer resistance. Surprisingly, the mole rats have high levels of oxidative stress and relatively short telomeres, yet they are extremely resilient when subjected to cellular stressors and appear capable of sustaining both their genomic and protein integrity under hostile conditions [56]. The resistance of mole rats to oxidative stress suggests resistance neurological damage. Hypoxic stress by nutrient oxygen deprivation in hippocampal slices of naked mole rat shows that neural tissue is resistant to nutrient oxygen deprivation [56] and likely resistance to AD toxicity. Neuregulin-1 (NRG-1) signaling, critical for normal brain function during both development and adulthood, is sustained throughout development and adulthood in mole rat [57]. Moreover, mean lifespan strongly correlated with levels of NRG-1, and its receptor, linking lifespan and NRG-1 levels. Neuregulin becomes a candidate target molecule for modulation, and the mole rat, a model organism for AD research.

## 9. Immunological Therapy and Innate Immunity

The major focus of Alzheimer's research is the attractive immunological therapeutic intervention approach to AD. Over 25,000 articles report the progress and perils of immunotherapy in treatment of AD as the focus of pharmaceutical drug discovery. Like induction of stress response to combat the disease challenge, induction of the immune response activates defenses to intervene in AD. Multiple reviews are available on the topic, and only a brief description of this valuable therapeutic approach is included here. An immunological solution has proven to be elusive, complex, costly, and ineffective so far, as the studies of the last decade reveal. Lessons learned include the discovery that although immunization of Amyloid $\beta$ (A$\beta$) peptide could protect and reverse amyloid pathology in animal models, in human trials, although immunotherapy did clear amyloid plaques, the clearance did not show a cognitive benefit effect in AD patients [58]. The amyloid hypothesis, as a target for AD immunotherapy, is at the crossroads [59]. Hope for (A$\beta$) vaccines remains, since a subset of patients with antibody titers in the active vaccine study, showed signs of

cognitive stabilization [60]. Adverse effects resulted in the discontinuance of human trials in an active vaccine study including meningoencephalitis with AN1792, vasogenic edema, and microhemorrhages with bapineuzumab, and uncertain results of cognitive benefit using passive (A$\beta$) immunotherapy in a genetic subgroup carriers of the APOE 4 gene [61]. New generation vaccines against (A$\beta$) peptide, and tau protein, may avoid adverse side effects, and slow progression of cognitive loss. The further refinement of AD DNA epitope vaccines is another immunological approach with promise for clinical trials administered preferably in preclinical stage individuals identified by validated AD biomarkers [62]. Unfortunately, agreement on the underlying cause(s) of AD is not established nor is the optimal immunological target(s).

An alternative immune therapy approach is the activation of innate immune function conserved throughout evolution, present in ancient organisms, and inducible in humans that does not require the knowledge of the causative agent of AD; rather activates a generalized resistance state. The preserved ancient immune T-cell immunoregulator, the CDR1 peptide of sharks, elicits an immune response in higher organisms and humans. The CDR1 peptide is involved in homeostasis, immunoregulation, response to infection, and reversal of the negative effects of immunosenescence on normal TH1 and TH2 T-cell subsets [63]. The TCR peptide itself restores balance between TH1 and TH2 and stimulates cells remodeling defective heart tissue implicating a role for immune system in cardiac repair [64]. The reversal of immunosenescence may directly impact the vulnerability of elderly to AD, or even provide repair after AD onset.

Another ancient immune factor is "the unmethylated CpG motifs," found to be prevalent in bacterial but not vertebrate genomic DNAs [65]. Oligodeoxynucleotides containing CpG motifs activate host defense mechanisms leading to innate and acquired immune responses. The recognition of CpG motifs requires the toll-like receptor. CpG-induced activation of innate immunity protects against lethal challenge from a wide variety of pathogens and has therapeutic activity in murine models of cancer and allergy. CpG motifs also enhance the development of acquired immune responses for prophylactic and therapeutic vaccination [65] and may boost immune function in AD vaccinations.

## 10. Stress Response Activation: Timing and Delivery

The optimal timing of intervention with alternative trigger induction strategies, intuitively, is prior to the onset of disease in known vulnerable candidates (early onset genetic predisposition, the elderly, prior history of brain damage), or in the early phases when there is detection of AD biomarkers, as is the preferred treatment population for all AD interventions. It is easier to prevent damage, rather than repair damage. However, there is promise for intervention in later stages of AD for delay of progression, or even reversal using triggers of stress resistance by upregulation of tolerance and rejuvenation after damage has occurred in other disease models, as outlined in the references presented above, for use in all stages of AD progression to delay progression or even reverse symptoms.

In theory, the delivery of mimetics of stress resistance triggers may be by oral, venous injection, or intranasal, since these delivery modes have been used to activate protective and rejuvenation response in rodent models, and in some cases to treat inflammatory human diseases. Especially promising is the use of intranasal delivery in neurodegenerative diseases and stroke [66]. Direct access to the damage brain tissue is attractive and may avoid other potential adverse effects by system-wide treatment.

From the above discussions, the theoretical benefits of the stress response triggers after disease onset include (1) the upregulation of protective mechanism to restore protein structure, using the inducers of chaperone proteins HSP's; (2) reduction of the increased inflammatory response to the disease states, and oxidative damage cascades, using hibernation like opioid mimetics, innate immune triggers, and endogenous antioxidant element triggers, to protect against further damage; (3) restoration of metabolic homeostasis, proteostasis, and antioxidant protection with the exercise mimetic, AICAR [43]. Cognitive function requires rejuvenation and repair, reduction of cell death, and induction of Nerve Growth Factors found in cells after AICAR treatment.

Theoretically, the vision is that induction of stress resistance will delay or stop the progression of the disease and even restore cognitive function. More than one trigger may be required, and/or the strategy of induction of stress resistance may be a valuable addition in genetic subpopulations resistant to immune therapies. Lessons learned from history teach us that theory and practice do not always coincide. Until appropriate controlled human clinical trials are explored and analyzed, the actual benefit of the proposed strategy remains unknown.

## 11. Conclusion

Mimetics of chemical and environmental stress can provide valuable activation of protective pathways with potential in intervention in the pathologies of AD now. The advantages of the activation of stress resistance as alternative strategy include availability, without new drug development, and, in some cases, triggers are already in human use to treat other metabolic, ischemic, and inflammatory disease conditions and pathologies. There is real hope for multiple options in AD intervention drugs presently in testing, alone or in combination with other therapies, especially in genetic subpopulations resistant to immunotherapy or other approaches.

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*Review Article*

# Neuroprotection and Neurodegeneration in Alzheimer's Disease: Role of Cardiovascular Disease Risk Factors, Implications for Dementia Rates, and Prevention with Aerobic Exercise in African Americans

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Prevalence of Alzheimer's disease (AD) will reach epidemic proportions in the United States and worldwide in the coming decades, and with substantially higher rates in African Americans (AAs) than in Whites. Older age, family history, low levels of education, and $\varepsilon 4$ allele of the apolipoprotein E (APOE) gene are recognized risk factors for the neurodegeneration in AD and related disorders. In AAs, the contributions of APOE gene to AD risk continue to engender a considerable debate. In addition to the established role of cardiovascular disease (CVD) risk in vascular dementia, it is now believed that CVD risk and its endophenotype may directly comediate AD phenotype. Given the pleiotropic effects of APOE on CVD and AD risks, the higher rates of CVD risks in AAs than in Whites, it is likely that CVD risks contribute to the disproportionately higher rates of AD in AAs. Though the advantageous effects of aerobic exercise on cognition is increasingly recognized, this evidence is hardly definitive, and data on AAs is lacking. In this paper, we will discuss the roles of CVD risk factors in the development of AD and related dementias, the susceptibility of these risk factors to physiologic adaptation, and fitness-related improvements in cognitive function. Its relevance to AD prevention in AAs is emphasized.

## 1. Introduction

Although anticholinesterase therapies have greatly improved symptomatic treatment of AD, they have not been demonstrated to significantly slow disease progression. Excess morbidity and mortality from AD continue to generate enormous economic burden on families and on the United States. Preservation of intellectual dexterity among those showing earliest symptoms of AD may ameliorate the physical, emotional, and economic burden associated with the disease, and that is an important public health goal.

A promising evidence-based and relatively side-effect free lifestyle approach is emerging as an alternative or adjunct to anticholinesterase therapy. Specifically, aerobic exercise training has been demonstrated to improve cognitive function (Figure 1). Though, the effect sizes for these studies were surprisingly large, and the results fairly consistent, however, the sample sizes were small and included mostly

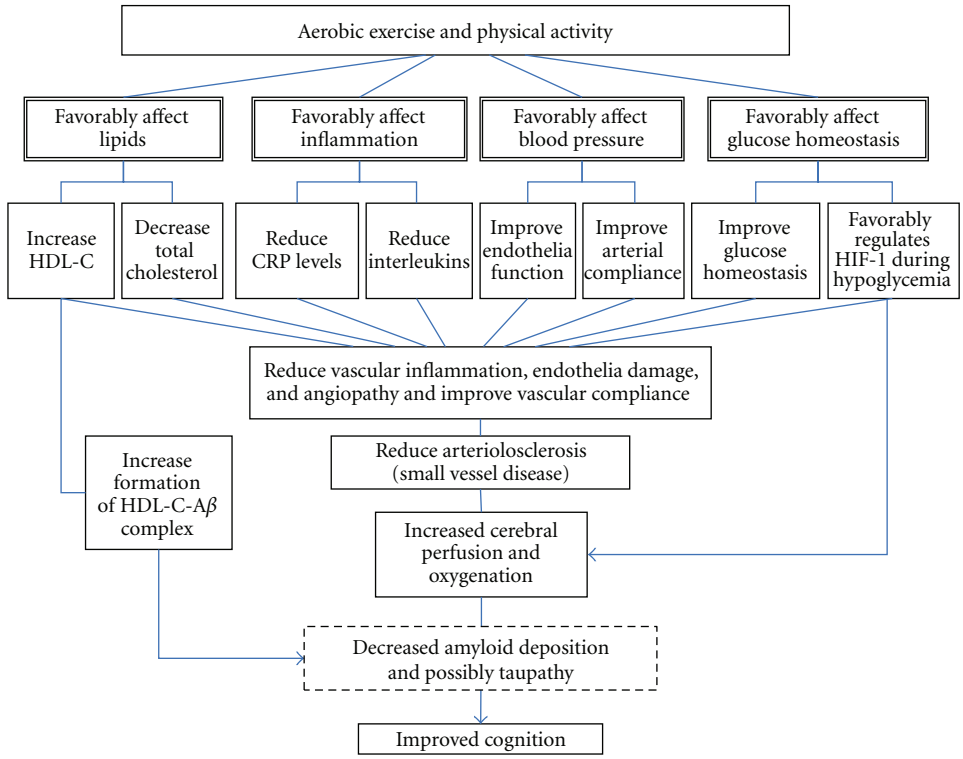


Figure 1: Aerobic exercise training and cognitive functions. Aerobic exercise increases HDL-C and subfractions; decrease total cholesterol, C-reactive protein, and interleukin-1; improves endothelia function and arterial compliance; improves glucose homeostasis and downregulates hypoxia.

Whites. Importantly, the mechanism by which an advantageous effect occurs is yet to be systematically examined. Remarkably, aerobic fitness can improve many of the putative AD risk factors such as high-density lipoprotein cholesterol (HDL-C), inflammation, and arteriolosclerosis. However, improvements in these risk factors have not been optimally explored as potential mechanisms by which aerobic training improves cognitive function in humans and AA in particular. Given that AAs (i) have higher incidence and prevalence of AD than Whites, (ii) have paucity of cross-sectional and lack prospective data on the beneficial effect of exercise on cognitive function and (iii) are more sedentary relative to Whites, in whom data show the beneficial effect of exercise, and therefore have more room for exercise-induced improvements in risk, it is relevant that the beneficial effects of aerobic fitness on neurocognitive processes is prospectively examined in this population.

## 2. Magnitude of Alzheimer's Disease Burden

Clinically, AD is a constellation of gradual decline in memory, other cognitive functions, behaviors, and activities of daily living leading to total dependency [1]. Pathologically, AD is a heterogeneous neurodegenerative disorder characterized by amyloid-beta plaques (A$\beta$), neurofibrillary tangles, inflammation, and neuronal loss. AD is the most common type of dementia constituting ~2/3rd of all late-life dementias and is estimated to affect 8 percent of persons age 65 years or older [2]. The prevalence of AD increased about 15-fold from 3 percent among individuals between the ages of 65, and 74 years to 47 percent for persons age 85 and older [3]. Also, the incidence of AD increased from 0.5 percent per year at age 65, to ~8 percent per year over age 85 [4, 5]. Without AD, hypertension, and other chronic age-related medical conditions, many older persons would remain relatively functional until late in life, contributing to society. That would reduce the nation's dependency ratio [6]. Based on 1999 estimates, the annual health care cost for AD was ~$100 billion [7]. Excluding ~$202 billion in uncompensated care by ~15 million families and caregivers, total payments in 2011 for health care, long-term care, and hospice services for people aged ≥65 years with AD and other dementias were estimated to be $183 billion [8]. Given this staggering cost and the projected increase in elderly population by the year 2050, identifying effective mechanisms to ward off structural and functional declines of AD is an important public health goal.

## 3. AD in African Americans

The incidence and prevalence of AD is higher in AAs than Blacks from Sub-Sahara African and compared to persons of European descent. In spite of this statistics, the disease is understudied in AAs [9]. In the Multi-Institutional Research on Alzheimer's Disease Genetic Epidemiology (MIRAGE) study led by Farrer et al., the adjusted cumulative risk of

Table 1: Number of deaths, population, and rate of death per 100,000 with underlying or contributing cause coded as dementia by division and race in persons aged 65 and over: United States 1999–2004.

| Division | Race | Death 65y+ | Population 65y+ | Crude rate 65y+ | Age adjusted rate 65y+ |
|---|---|---|---|---|---|
| New England | Black or African American | 1,683 | 340,854 | 494 | 574 |
| | White | 77,719 | 10,875,302 | 715 | 633 |
| Middle Atlantic | Black or African American | 10,145 | 3,119,034 | 325 | 362 |
| | White | 155,700 | 28,965,773 | 538 | 492 |
| East North Central | Black or African American | 17,106 | 2,836,888 | 603 | 671 |
| | White | 219,113 | 31,040,245 | 706 | 664 |
| West North Central | Black or African American | 3,476 | 493,880 | 704 | 757 |
| | White | 115,938 | 14,864,882 | 780 | 687 |
| South Atlantic | Black or African American | 37,538 | 5,553,447 | 676 | 731 |
| | White | 240,812 | 36,129,123 | 667 | 676 |
| East South Central | Black or African American | 11,422 | 1,798,196 | 635 | 625 |
| | White | 75,592 | 11,093,302 | 681 | 721 |
| West South Central | Black or African American | 12,364 | 2,158,225 | 573 | 597 |
| | White | 118,490 | 18,293,681 | 648 | 676 |
| Mountain | Black or African American | 1,242 | 232,688 | 534 | 688 |
| | White | 78,387 | 12,005,553 | 653 | 687 |
| Pacific | Black or African American | 8,352 | 1,283,968 | 650 | 725 |
| | White | 181,815 | 25,078,447 | 725 | 683 |
| US total | Black or African American | 103,328 | 17,799,544 | 581 | 628 |
| | White | 1,263,566 | 188,249,878 | 671 | 647 |

dementia in the first degree relatives of probands with AD in AAs was approximately twice that of a similar White sample. According to reports from the Indianapolis-Ibadan Dementia Project, the rates of AD and dementia in Yoruba (an ancestral population in Nigeria) are less than half the rates in AAs [10], suggesting possible contributions from the environment.

To better discern the relatively high rates of AD in AAs, a number of studies have compared the prevalence and incidence of AD and related disorders across populations in the US. Whereas a faster rate of cognitive decline in Mild Cognitively Impaired (MCI) AAs than in non-AA was observed in one study that used a community-based sample [11], others found no evidence of racial disparities in cognitive trajectories of MCI [12, 13]. However, in AAs compared to Whites, a significantly slower rate of cognitive decline was reported once AD begins [13, 14]. For example, using age and education adjusted growth curve approach to estimate individual paths of change in global cognition, Barnes reported that older AAs had a lower level of global cognition at baseline and declined at ∼25% slower rate compared to Whites [14]. In another study that examined the severity of AD at the time of presentation to the medical establishment among different ethnic groups in the US, minority persons (including AAs) compared to Whites tended to exhibit a more severe profile of AD at the time of presentation [15]. Despite such relatively slower rate of AD progression, AA MCI and incident AD patents experienced greater decline in body mass index (BMI) compared to normal controls [16]. While the biologic explanation for the lower rates of cognitive decline in AAs needs further elucidation, an enriched social network has been proposed as a possible explanation [17]. Collectively; a higher incidence and greater rate of cognitive decline in MCI and AD-afflicted AAs, delayed diagnosis, lower rate of cognitive decline once AD occurs together with an accelerated weight loss suggest that the overall prevalence of AD in this population will reach epidemic proportions in the coming decades. Decreased overall wellness and increased health disparity are notable consequences. Given the effects of socioeconomic variables and access to health care on these important health indicators, such consequences may become blurred by regional variations. In support of this view, we recently reported that racial differences in AD or dementia mortality varied by regions in the United States (Table 1) [12, 18]. A fundamentally important implication of these observations is that other factors at the environment or genetic level may contribute to higher incidence and prevalence of AD in AAs than in Whites. Increased CVD risks and low levels of physical activity may explain some of these differences.

At the genetic level, APOE gene is the most consistent nondeterministic genetic risk factor for AD. Its contributions to AD risk are graded across alleles ($\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$), with $\varepsilon 4$ conferring the highest risk [19, 20]. While many believed that the contributions of the $\varepsilon 4$ allele to AD risk are similar across populations [21, 22], others have reported a lower associated AD risk in AAs than in Whites [23, 24]. Using pooled samples from the MIRAGE, Alzheimer's Disease Neuroimaging Initiatives (ADNI), Canadian Study on Genetics of Alzheimer's Disease Association (GenADA), and National Institute on Aging-Late-Onset Alzheimer's Disease Family Study (NIA-LOAD) data, we reported that the presence of $\varepsilon 4$

allele significantly and exponentially associated with AD in AAs in a dose-dependent manner. However, the odds ratio estimates in $\varepsilon 4$ carriers showed lower rates of AD in AAs compared to Whites (63.1 percent versus 67 percent). Conversely, we also observed a higher occurrence of $\varepsilon 4$ in AA controls than White controls (40.1 percent versus 29.1 percent), respectively [25]. These suggest that the $\varepsilon 4$ allele of the APOE gene may interact with other risk factors to cause a differential AD risk in AAs compared to Whites. Interestingly, AAs also have increased CVD risks such as hypertension, diabetes, and hypercholesterolemia. Evidently, key interactions of APOE gene with CVD risk such as lipids, inflammation, glucose homeostasis, and lifestyle factors in these populations must be considered [26]. Such factors may lend themselves to interventions capable of attenuating AD risk in AAs and other populations at risk.

At the environment level, growing evidence indicates that aerobic fitness can reduce AD risk in predominantly White samples. However, these advantageous effects of exercise are yet to be validated in a relatively more sedentary AA sample [27]. Given the higher rates of AD in AAs than in Whites and the lack of substantive differences in AD neuropathology [28, 29], it is likely that AA mild AD patients will also benefit from the advantageous effects of aerobic fitness. In addition to the public health imperative, such intervention may ameliorate the physical, emotion, and economic burden associated with AD. All of these effects will benefit society at large.

## 4. Rationale for Dementia Prevention

Whereas, it is established that the preservation of neurocognitive function among those showing earliest signs and symptoms of AD can attenuate the burden associated with the disease; unfortunately, this benefit and the national goals of Healthy People 2010 cannot be realized without an efficient AD prevention strategy. Moreover, while medical treatment after disease onset may reduce disease progression and mortality, eventually, increases in disease prevalence will substantially escalate total disease burden and healthcare cost for the population. Though the current approach to symptomatic treatment of AD may not be cost-effective in populations with excessive rates of disease such as AAs, a low-cost low-risk intervention strategy with dual applicability for primary and secondary prevention is likely to be advantageous.

The goal of this paper, therefore, is to enhance scientific discuss on the role of CVD risk in the development of AD and related dementias and to add clarity to the clinical utility of fitness adaptation in preventing AD in those at risk. However, significant uncertainty in disease progression from prodromal to symptomatic AD raises an important question of whether intervention should be directed at the fully characterized MCI or AD clinical phenotypes. Because of the present impracticality of reversing neuronal death underlying the AD phenotype, interventions are likely to yield the most benefit if initiated at the earliest possible stage as in pre-MCI. Indicators of such timely intervention may include notable endophenotype such as decreasing cerebrospinal fluid (CSF) levels of A$\beta$ that precede the emergence of the MCI clinical phenotype. If confirmed in randomized clinical trials, aerobic fitness can become an effective public health tool to combat AD risk. Such a low-cost low-risk effective strategy is likely to reduce the burden of disease and optimize the well-being of older adults at increased AD risk.

## 5. Cardiovascular Disease Risk in AD Development

Stroke and Alzheimer's type dementia increase at comparable rates with advancing age. Atherosclerosis, hypertension, diabetes mellitus, and lipids are major CVD risk factors shown to be associated with AD [30]. Recently, Arvanitakis and colleagues reported an association of diabetes with semantic memory impairment in both Blacks and Whites [30]. The Rotterdam population-based prospective study that examined approximately 8000 subjects over age 55 for the frequency of lifetime risk of dementia and its subtypes, including AD, showed an increase in the prevalence of atherosclerosis in both vascular dementia and AD [31]. Also, compilation of autopsy reports on AD brains indicate, that approximately 60–90 percent of the cases exhibited variable cerebrovascular pathology synonymous with CVD [28–32]. In AD cases ascertained by the presence of amyloid angiopathy, endothelial degeneration, and periventricular white matter lesions at autopsy, Van Nostand showed that ~1/3rd had evidence of cerebral infarction [33]. However, in a study to examine the relationship of important AD intermediate phenotype such as differences in brain volume, hippocampal volume and cerebrovascular risk factors, and APOE4 among MCI subtypes, He and colleagues found CVD risk factors to be more closely related to nonamnestic MCI and vascular dementia; though emphasized that the biological differences between amnestic (AD group) and nonamnestic (presumed vascular etiology) were very subtle [34]. Given these observations, it is possible that CVD risks plays a greater role in cognitive decline in older AAs compared to Whites. In support of this view, Brickman et al. demonstrated more severe white matter hyperintensity (WMH) burden in AAs and Hispanics compared to Whites [35]. In particular, vascular disease was associated with relatively smaller brain volume and higher WMH burden in AAs. Others have also demonstrated greater degree of psychomotor impairment, a surrogate for higher cerebrovascular burden in AAs than in Caucasians [36]. Collectively, these reports indicate that CVD risk factors may also influence cognitive loss, particularly in AAs who suffer a greater burden of CVD risk and related brain pathology.

Regardless of whether increased CVD risk burden culminates into vascular dementia, enables or directly promote AD pathology [37–42], with or without interactions with age-associated decline in health status [43], interventions directed at reducing CVD risk factors may attenuate declining cognitive dexterity especially in older AAs. Despite the evidence showing a higher degree of CVD risks and cerebrovascular pathology in AAs, data is lacking on whether aerobic fitness-induced reduction in CVD risk can concomitantly reduce AD risk in this population. Given that AAs suffer a high CVD-related morbidity, they are likely to benefit from CVD risk reduction measures. Collection of prospective data on putative CVD mediators of AD and their

susceptibility to fitness adaptation will elucidate its clinical utility in ameliorating AD risks in AAs and other populations.

## 6. Mechanisms by Which Cardiovascular Disease Risk Can Influence AD

*6.1. Association of Total Cholesterol with AD Risk.* Disorder of brain cholesterol metabolism has been associated with all principal pathological features of AD such as synaptic transmission [44], amyloid [45], and tau pathology [44]. Lipids and lipid peroxidation products have important roles in the homeostasis of the central nervous system [46]. In animal and in vitro studies, Golde and colleagues showed that overexpression of cholesterol resulted in the formation of amyloid $\beta$ and contributed to the degradation of neurons and subsequent cognitive impairment [47]. Also, lipid transport genes and vascular changes associated with peripheral dyslipidemia have been associated with an increased risk of AD. This indicates that lipids may be involved in the pathogenesis of neurodegeneration and related dementias. Alternatively, lack of cholesterol supply to the neurons via lipoprotein transport may cause failure of neurotransmission and synaptic plasticity [48]. However, because almost all brain cholesterol is a product of local synthesis, with brain blood barrier efficiently protecting it from exchange with lipoprotein cholesterol in the systemic circulation [49], serum cholesterol may not accurately reflect the related AD risk. Moreover, the bimodal relationship of serum cholesterol with health may contribute to the inconsistencies of reports on the association of cholesterol with cognitive health, especially when the protective influence of HDL-C is not considered.

*6.2. Association of High-Density Lipoprotein Cholesterol with AD and CVD Risk.* HDL-C is an important risk factor for CVD [50, 51]. As with CVD risk, the contribution of HDL-C to AD risk is increasingly recognized. HDL-C functions to both keep its lipid components soluble and also provide an efficient mechanism for their transportation through plasma and to or from the tissues. Low HDL-C, together with suboptimal transport system in humans, results in gradual deposition of lipid (especially cholesterol) in tissues causing arteriolar narrowing and chronic cerebral oxygen deprivation [52].

*6.3. HDL-C Is the Predominant Lipoprotein in Human Brain Circulation, and Its Low Levels Have Been Associated with Impaired Memory [53–55].* For example, Wolf and colleagues recently showed that low levels of HDL-C and not LDL or total cholesterol levels were associated with hippocampus atrophy in aged humans [56]. Unlike total cholesterol, HDL-C brain level correlates with its plasma concentration. This evidence suggests that low levels of HDL-C may play an important role in AD risk. Beyond the direct effect of low HDL-C on arteriolosclerosis, high HDL-C may conversely influence AD risk in three other important ways: (i) mediation of reduced inflammatory cytokines which is central to arteriolar narrowing; (ii) through its interaction with A$\beta$ to form soluble HDL-C-A$\beta$ complex (Figure 2); (iii) its antioxidant property.

*6.4. Evidence of Anti-Inflammatory Effects of HDL-C.* In support of HDL-C anti-inflammatory effects, Cockerill and colleagues showed that, in physiological concentration, isolated plasma HDL-C inhibited tumor necrosis factor-$\alpha$ (TNF-$\alpha$) or interlekin-1 (IL-1) and reduced leukocyte adhesion molecules in a concentration-dependent manner (Figure 2) [57]. Others have reported increased markers of inflammation with low HDL-C levels [58]. Therefore, as the predominant lipoprotein in the brain circulation, the anti-inflammatory effects of high HDL-C may play an active role in reducing vascular inflammation and arteriolosclerosis of the cerebral circulations. This may enhance brain oxygenation and preserve neurocognitive dexterity.

*6.5. Evidence of Antiamyloid Deposition Effects of HDL-C.* The interaction of HDL-C with A$\beta$ is consistent with its neuroprotective effects. For example, HDL-C attenuates the aggregation and polymerization of A$\beta$ protein (Figure 2) [59]. Using thioflavin T fluorescence, Olesen and Dagø showed that HDL-C reduced amyloid formation in vitro. Additionally, the association of HDL-C with A$\beta$ was also recently demonstrated by Koudinov et al. who isolated HDL-A$\beta$ complexes from CSF [60]. More support for the direct effects of HDL-C on A$\beta$ was evidenced by studies showing that A$\beta$ mediated the cellular uptake of lipoproteins [61], and that HDL-C induced increases in the cellular degradation of A$\beta$ in cultured microglia [62]. Its neuroprotective property against A$\beta$ was also demonstrated by Farhangrazi et al., who showed that the neurotoxic effect of A$\beta$ in cortical cell cultures became attenuated in the presence of high levels of HDL-C [63]. It is therefore likely that HDL-C exerts a significant antiamyloid effect that may be susceptible to lifestyle alteration.

*6.6. Evidence of Antioxidant Effect of HDL-C.* Growing evidence suggests that oxidative damage is implicated in neuronal degeneration that occurs in AD brains [64, 65] High-plasma HDL-C particles can also exert antioxidant activity and have the capacity to protect low-density lipoprotein (LDL) against oxidative stress [66]. Though the exact mechanism by which HDL-C exerts antioxidant effects needs further clarification, its role as a transporter of enzymes exerting antioxidative activity such as paraoxonase (PON) [67], platelet-activating factor acetylhydrolase (PAF-AH) [68] and lecithin-cholesterol acyltransferase (LCAT) [69] must be noted. Moreover, intrinsic antioxidative property of HDL-C subfraction is deficient in the presence of low HDL-C phenotype and amplified by low number of circulating HDL-C particles. Indeed, this dysfunctionality is closely related to elevated oxidative stress evidenced by breakdown products of arachidonic acid such as plasma isoprostane.

In summary, given the effects of high HDL-C levels on the biochemical properties of A$\beta$ and its antioxidant property, it is likely that HDL-C plays a direct role in brain amyloid deposition and AD risk. Because high HDL-C can reduce inflammation, enhance lipid metabolism, and therefore reduce arteriolosclerosis and enhance brain perfusion, it is likely to be important for optimal neurocognitive function. Fortunately, HDL-C is susceptible to the effects of aerobic

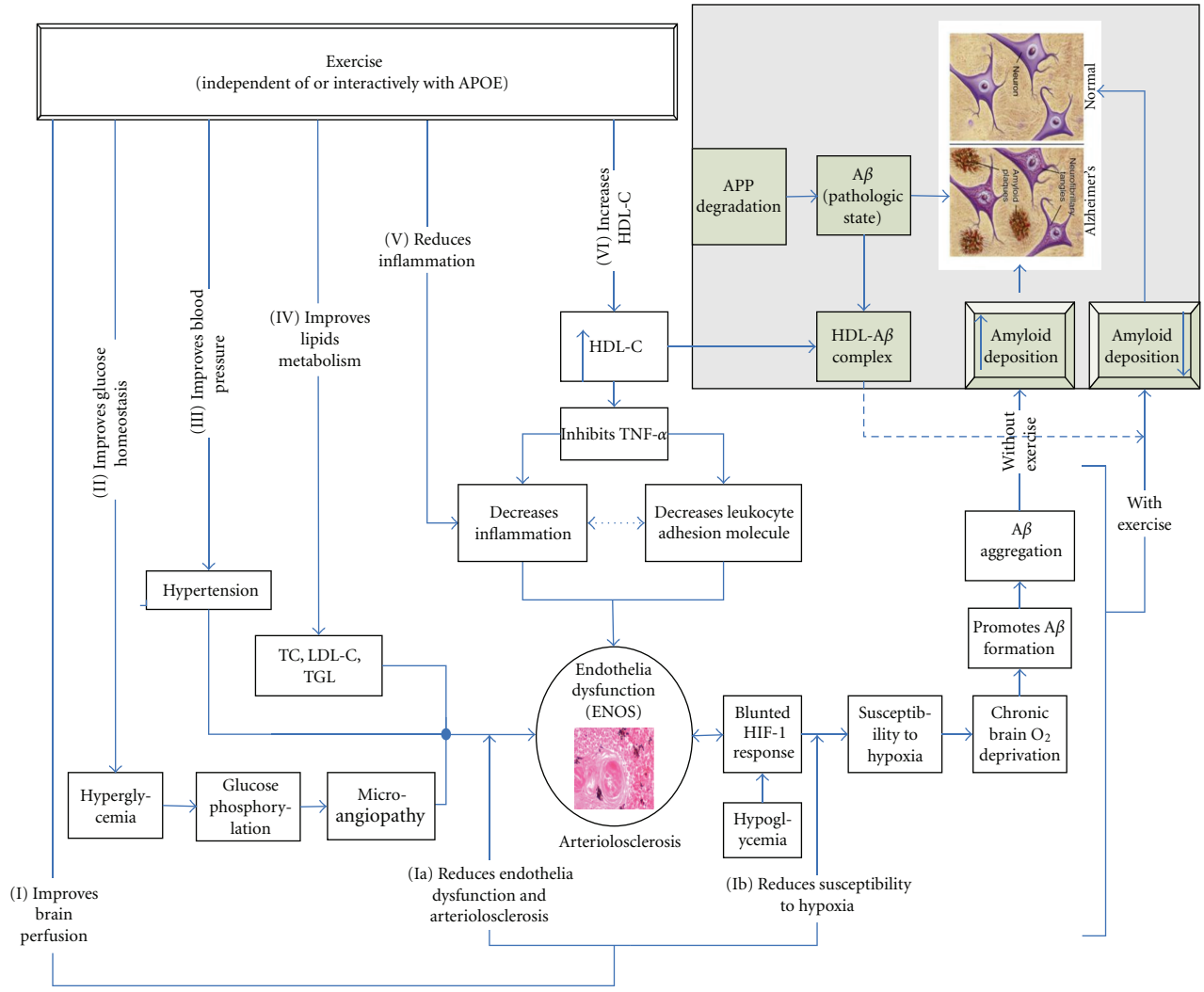


Figure 2: Interaction of HDL-C with AD risk factors. Relationships of exercise to prevention of intracerebral amyloid deposition.

exercise training. Our own aerobic fitness data indicated that a 6-month aerobic exercise-training can improve protective HDL-C large particle size in AAs [70]. Whether this improvement translates into improvement in cognitive function is the subject of our ongoing investigations.

*6.7. Association of Inflammation with AD Risk.* Though considerable uncertainty exists on the exact role of the inflammation in AD, many studies have documented the association of inflammatory markers such as CRP and IL1a with AD. The role of inflammation has become even more evident with recent studies on microglia. Microglia, a distinct population of brain-resident macrophages, is indicative of ongoing chronic inflammation in AD. In support of anti-inflammatory role of microglia, Minagar and McGeer demonstrated its activation in regions of the brain showing AD pathology [71, 72]. Building on earlier observations, Frank et al. recently examined the association of inflammation with the neuropathology of AD and showed that microglia are present in close association with aggregated types of Aβ plaques and around neurofibrillary tangles [73]. Frank et al. also showed that microglia-derived factors including reactive oxygen species and tumor necrosis factor-α (TNF-α) are neurotoxic [73]. Neuronal damage by microglia can also occur when activated microglia and reactive astrocytes surrounding intracellular deposits of Aβ protein initiate an inflammatory response [74]. Often, this type of response is characterized by local cytokine-mediated acute phase response and activation of the complement cascade [74].

However, studies on the effects of anti-inflammatory agents on AD risk are inconclusive. For example, a retrospective study of long-term users of nonsteroidal anti-inflammatory drugs showed a lower incidence of AD in this population [75]. Conversely, recent clinical trials found no benefit to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [76, 77]. Since the actual dose, duration, and period of protective NSAID use are unknown, these negative results are hardly definitive. Further, many of these studies did not account for genetic mediators of inflammatory markers. Notable among such markers are interleukins and

C-reactive protein (CRP). We and others have shown that aerobic fitness either independently or interactively with its genetic mediators can reduce CRP level [78, 79]. Whether training-induced reductions in inflammation and CRP levels translate into improvements in cognitive performance has not been studied. In our currently ongoing pilot clinical trial, we will further delineate the role of inflammation in AD, its association with HDL-C and A$\beta$ protein, and whether exercise-related changes in inflammatory markers are associated with neurocognitive measures that are used in this study. Demonstration of concomitant reduction in inflammatory markers, with improvements in neurocognitive function after aerobic exercise training, will be important evidence supporting the role of inflammation in AD. This would be indicative of the susceptibility of AD risk factors to aerobic fitness.

## 7. Association of Hypertension with AD Risk

Growing evidence indicates a causal role of hypertension for cognitive decline of the Alzheimer's type dementia (Figure 2) [80, 81]. A few longitudinal studies have also emphasized a connection between high blood pressure in midlife and dementia in late life [80, 81]. Recently, Korf and colleagues reported an association of systolic blood pressure (SBP) and pulse pressure (PP) with medial temporal lobe atrophy (MTA), a hallmark of AD, in individuals with late onset dementia, especially when coexisting with white matter changes [82]. These reports indicate that CVD risk factors including hypertension may also influence AD risk. Our own data from the NHANES III support these observations. Though the optimal BP for cognitive performance remains poorly defined and evidence is emerging on the effects of CVD-related genes such as ENOS and ACE on AD, the combined effects of hypertension and genetics on neurocognitive function need clarity. Like many other CVD-related AD risk, considerable evidence suggests that fitness adaptation can reduce blood pressure [83–85]. However, whether a concomitant improvement in neurocognitive performance occurs with aerobic fitness-related improvements in blood pressure has not been examined. Even if very small cognitive benefit accrues from blood pressure reduction, substantial gains can be realized, given the relatively high prevalence of hypertension in the United States and in the World.

## 8. Association of Hypoxia and Glucose Homeostasis with AD Risk

Neurons are highly vulnerable to impairments of oxygen homeostasis because of their singular dependency on oxygen. Though the human brain averages about 2 percent of body mass, it utilizes 15 percent of cardiac output and 20 percent of respiratory oxygen uptake. In neural cells in primary culture and in the hippocampus using in vivo models, both cycloo-2 (COX2) and presenile-1 (PS1) are induced after only about 5 minutes of hypoxia [86, 87]. Cell cultures and transgenic models also suggest an interactive relationship of hypoxia with microglia activation, neuroinflammation, reduced neuronal function, and apoptosis [88–90]. These reports are indicative of the independent and collective roles of CVD risk factors and, importantly hypoxia in AD risk.

Changes in brain glucose metabolism are associated with AD [91, 92], and the upregulation of glucose metabolism has been demonstrated to activate the transcription of hypoxia inducible factor (Figure 2) (HIF-1) [93]. HIF-1 is a heterodimeric transcription factor comprised of two subunits, HIF-1$\alpha$ and HIF-1$\beta$. In normoxic state, the binding and transcription of hypoxia-inducible genes do not occur [94]. HIF-1 mediates the adaptation of cells to hypoxia and hypoglycemia by upregulating genes involved in glucose transport and glycolysis [93]. Blunted HIF-1 response to hyroxia has been shown to promote A$\beta$ formation and changes in glucose metabolism. Together, this evidence suggests that inflammation, acting in concert with HIF-1, and glucose metabolism may play an active role in brain cellular damage and ultimately AD. Fortunately, fitness adaptation can enhance glucose uptake, increase cerebral perfusion and possibly favorably regulate the activation of HIF-1. However, there is no randomized, controlled experiment linking aerobic fitness to improvements in these intermediate phenotypes or neurocognitive function. Large-scale clinical trials are needed to determine whether fitness-related improvements in brain perfusion are effective intervention strategies to reduce AD risk.

## 9. Exercise Effects on Cognitive Function

*9.1. Fitness Training Is Associated with Improved Cognitive Health in Cross-Sectional and Few Prospective Studies.* Cross-sectional [95, 96], longitudinal [97], and meta-analyses have demonstrated that improvements in cardiovascular fitness can improve cognitive function in humans [98, 99]. For example, Larson recently showed a <3 times/week exercise to be related to increased risk of AD compared to >3 times/week exercise [100]. Others have reported an inverse relationship of AD with the number of physical activities performed. [96] In a study of leisure-time physical activity during midlife and dementia, Rovio et al. reported a reduced risk of AD in those with higher levels of physical activity [95]. These studies suggest a significant association of physical activity with later reduction in neurocognitive function and dementia. Notwithstanding the mostly beneficial effects of exercise observed in the majority of studies, limitations such as self-reported data; failure to distinguish between aerobic and noaerobic activities; failure to assess exercise duration, intensity, and frequency; differences in the volume of exercise that is beneficial likely resulted in significant variability among studies.

To obviate the limitation inherent in cross-sectional studies, a few prospective studies have examined the effects of fitness adaptation on memory. Using meta-analyses of 18 published studies, Colcombe and Kramer found a beneficial effect of fitness training on an array of neurocognitive process in nondemented older adults [99]. In a ~7-year prospective study of 5925 older women, Yaffe et al. demonstrated a 37% reduction in the odds of cognitive decline in 3rd quartile compared to 1st quartile of physical activity [101]. In another prospective study, Barnes and colleagues reported better

cardiorespiratory fitness at baseline to be associated with less cognitive decline at ~6-year followup [102]. A recent 24-week randomized placebo control trial of an unsupervised physical activity intervention study in MCI-like subjects by Lautenschlager et al. revealed an improvement of 1.0 points in ADAS-cog for exercisers, and a deterioration of 1.3 for controls yielded a total of 2.3 point difference between the intervention and control groups over 6 months. Interestingly, the cognitively beneficial effects of aerobic fitness remained at 18-month followup [103]. Though these prospective studies add substantially to the current knowledge and the directionality of the relationship of aerobic fitness with neurocognitive function, data is lacking on AAs. Importantly, a more rigorous randomized controlled trial in MCI patients is needed to establish causality and to clearly delineate the overall volume of exercise that is beneficial. In spite of the skepticism on the relatively large aerobic fitness-related effect size reported in many studies, the multiple levels at which exercise can influence AD risk support such observations.

## 10. Mechanism by Which Exercise Influences Neurocognitive Function

Mechanism by which aerobic fitness affects neurocognitive health is yet to be clearly elucidated. Despite the evidence showing an association between exercise engagement and improvements in AD biomarkers in cognitively normal older adults [104] and reports of increased aerobic fitness-related increases in brain volume in some studies [105–107], the underlying biological mechanism for these effects needs further clarifications. Given the available evidence, it appears that the effects of exercise on neurocognitive function are mediated through several important pathways. Dyslipidemia, especially low HDL-C levels, inflammation, deranged glucose homeostasis, and endothelia dysfunction are precursors of arteriolosclerosis, decreased cerebral perfusion and cerebral oxygen deprivation, all of which may increase AD risk [108, 109]. Aerobic fitness can increase HDL-C, reduce inflammation [78], improve glucose homeostasis [110], and reduce arteriolosclerosis. Because these benefits can enhance brain perfusion and improve brain oxygenation, likely benefits include reduction in AD risk [111] (Figure 1). Our own analysis of the data from NHANES III supports the advantageous effects of high levels of HDL-C (Figures 3(a) and 3(b)). Because exercise can cause reduction in stress hormone levels known to impair cognitive function [112]; promote neurotrophic changes, nerve cell regeneration, and neurotransmitter repletion, all of which may enhance cognitive performance [113, 114], these effects are likely involved in the mechanism by which aerobic fitness affects neurocognitive function. Since the evidence suggests that exercise can increase solubility of A$\beta$ through increases in HDL-C [62] and favorably regulate hypoxia inducible factor (Figures 1 and 3), these effects may represent alternative important mechanism by which exercise exerts its advantageous effect on neurocognitive function. Training-induced improvements in these putative AD risk factors may precede more distal effects of fitness adaptation such as increased activity in the frontal and parietal regions of the brain and increased gray matter volume in the frontal and superior temporal lobe reported by Colcombe and Kramer, respectively [115, 116]. Collectively, these observations indicate that aerobic fitness may attenuate neurocognitive loss in humans.

## 11. Limitations of Knowledge on the Effects of Exercise on Neurocognitive Function

While most of the studies on the effects of aerobic fitness on cognition are indicative of its beneficial effects, few limitations of these studies must be pointed out. First, most have not used a standardized exercise protocol, none used randomized controlled design in MCI or mild AD patients. While the evidence supports an overlap of CVD risk with AD risk and the responsiveness of CVD risk factor to fitness adaptation, most of the intervention studies thus far have not explored CVD risk reduction as the mechanism for improvement in cognitive performance. A prospective randomized controlled trial of aerobic fitness with biomarkers and neuroimaging will inform the establishment of causality, and help determine the volume of exercise that is beneficial. Notably, it will lay the groundwork for the determination of the role of genetics in aerobic fitness-related effects and the mechanism by which fitness affects neurocognitive function.

## 12. Apolipoprotein E Gene as a Modifier of AD Risk

*12.1. APOE Is a Risk Factor for AD.* The evidence suggests that the APOE gene, especially the $\epsilon 4$ subtype, is a major risk factor for sporadic and late-onset Alzheimer's dementia [117, 118]. There are three known common isoforms of APO (E2, E3, and E4) in humans encoded by the different alleles $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$. It acts as a receptor of ligands, signifying that intraneuronal APOE may be a mechanism by which APOE influences neuronal repair, regeneration, and survival. Further, APOE can interact with $\beta$-amyloid and tau proteins that are central to the pathogenesis of Alzheimer's dementia. Specifically, the presence of APOE lipoprotein in cerebral blood vessels laden with amyloid $\beta$-protein (A-$\beta$) [119] is indicative of the importance of Apoliporotein in the pathogenesis of AD.

*12.2. APOE Gene May Influence AD Risk through Its Effects on High-Density Lipoprotein Metabolism.* Similar to the role of the $\epsilon 4$ allele APOE gene in the pathogenesis of Alzheimer's dementia, its association with elevated lipid levels [120] and atherosclerosis have also been reported [121]. Genetic variation at the APOE locus can also influence atherogenesis through its effects on HDL-C subfractions. APOE affects the hepatic binding, uptake, and catabolism of several classes of lipoproteins associated with HDL-C subfractions [122, 123]. The $\varepsilon 2$ and $\varepsilon 4$ alleles of the APOE gene are associated with higher and lower HDL-C subfractions, respectively, among different ethnic subgroups and across regional boundaries [124, 125]. Together, these observations suggest that an individuals' genetic makeup, especially at the APOE, locus may interact with the environment to influence HDL-C levels. Because of the importance of apolipoprotein to HDL-C metabolism and its susceptibility to the influence of APOE

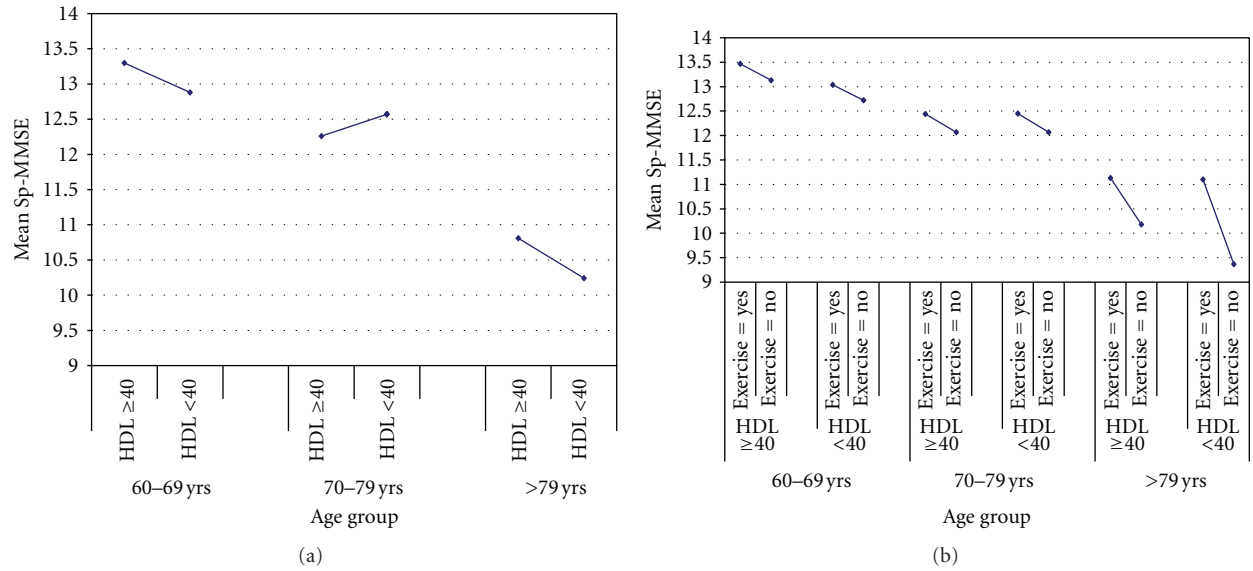


Figure 3: Adjusted mean short portable MMSE by HDL-C levels and aerobic exercise training.

gene, their combined role in the pathogenesis of AD should be of significant interest.

*12.3. African Americans: The Role of APO E in AD Risk.* Evidence from protein binding indicates that A$\beta$ interacts with APOE in an isoform specific manner, and fibril formation of A$\beta$ is enhanced by the presence of $\varepsilon$4 allele of the APOE gene. In its physiologic state, APOE is normally present in the brain in association with HDL-C-like particles. In view of the important role of the $\varepsilon$4 allele and its overrepresentation in AAs, a proportionately higher $\varepsilon$4-associated AD risk in AAs would be expected. However, some evidence suggests the converse.

The interaction of HDL-C with APOE provides a useful insight into the reduced $\varepsilon$4 allele-associated AD risk, and the slower rate of AD progression in AAs. Consistently, higher levels of HDL-C have been shown in AAs than elderly Caucasians [126, 127]. In the presence of HDL-C particle, Olesen and colleagues found no direct effect of APOE on amyloid formation [59]. This suggest that, though $\varepsilon$4 may increase the spontaneous amyloid formation of A$\beta$, HDL-C-bound A$\beta$ appear to decrease amyloid formation as a result of strong amyloid inhibitory effect of HDL-C. Alternatively, $\varepsilon$4 allele may influence amyloid formation by affecting the levels of HDL-C-like particles in the brain. Therefore, because AAs have relatively higher levels of HDL-C, it is possible that HDL-C interacts with APOE to reduce the $\varepsilon$4 allele-related AD risk and, importantly, lower the rates of disease progression in this population.

*12.4. Combined Effect of APOE Gene and Exercise Training on HDL-C in AAs.* Increased levels of HDL-C and $HDL_2$-C are the most significant changes in lipid and lipoprotein levels that occur following aerobic exercise training [128, 129] Results from exercise training studies show higher levels of HDL-C after exercise in most older Whites [130, 131]. Such highly variable responses to a standardized exercise training intervention may implicate genetic factors as contributors.

Across all adult age groups, habitual levels of physical activity are significantly lower in AAs than in Caucasian Americans for both men and women [132, 133]. A sedentary lifestyle among older AAs leads to obesity and higher triglycerides (TG) and LDL-C, but lower HDL-C [134, 135]. Conversely, exercise training can reduce TG and LDL-C and increase HDL-C. Following 10 weeks of aerobic exercise training, Doshi et al. reported an 8% reduction in cholesterol/HDL-C ratio in older AAs, independent of changes in body composition [136]. Conversely, a study in South African Blacks found no significant change in HDL-C levels after exercise training [137]. These studies suggest that exercise training may increase HDL-C levels in some AAs. Significant interactions with APOE genotype are one possible mechanism by which this can occur. Interestingly, our own standardized aerobic exercise training data showed fitness-related increases in the levels of HDL-C particle size and concentration in $\varepsilon$2/3 and $\varepsilon$4 AAs, though to a lower extent in $\varepsilon$4 carriers. Therefore, APOE and other genetic markers may account for some of the disagreements among studies.

In our currently ongoing pilot study, we will collect prospective data on APOE, HDL-C (particle size and concentration), other biomarkers, neurocognitive function, and neuroimaging. Data on the interactive effects of HDL-C, APOE, and aerobic exercise training on neurocognitive function, will be used to inform the power calculation for a full-scale clinical trial to determine the mechanism by which aerobic-fitness affects neurocognitive function.

*12.5. Summary of Current Knowledge, Gaps.* The evidence highlights the central role of CVD risk and chronic cerebral oxygen deprivation to neurocognitive health. Importantly, disorders of brain lipid metabolism are associated with all principal pathological features of AD such as synaptic

transmission [44], inflammation, amyloid [45], and tau pathology [44]. HDL-C is the predominant lipoprotein in human brain circulation, and its low levels can impair memory [53–55]. Unlike total cholesterol, its brain levels reflect blood level. Low levels of HDL-C is associated with hippocampal atrophy in aged humans [56] and therefore likely to be involved in the effects of lipids on cognitive function. Because HDL-C can also increase the cellular degradation of A$\beta$, and decrease A$\beta$-induced neurotoxicity in neural culture, it is likely that HDL-C also plays an important role in the biochemical properties of A$\beta$ amyloid formation, and AD. Aerobic exercise can increase HDL-C, reduce inflammation, improve glucose homeostasis, and enhance cerebral perfusion.

Cross-sectional and few prospective studies in predominantly normal Whites samples suggested that aerobic fitness can enhance cognitive function [96, 102, 116, 125, 138]. The outcome of these studies are hardly definitive, and the mechanism by which fitness adaptation affect cognitive function remains to be fully elucidated. Though we and others have shown that exercise can increase HDL-C (Figure 3), the effect of aerobic fitness-induced changes in HDL-C on preservation of neurocognitive function is yet to be examined. Further, whether these changes correlate with changes in cerebral glucose homeostasis is not known. Future studies must focus not just on CVD risk factors and brain infarcts, but also on its surrogates such as increased vascular resistance and chronic cerebral oxygen insufficiency with or without infarcts as well as decreased oxygenation associated with age-related decline in pulmonary function. The role of HIF in these cascades of events must also be considered.

Consistently, the APOE gene has been shown to influence both HDL-C metabolism and independently AD risk. In view of the susceptibility of HDL-C to aerobic fitness and the importance of APOE gene to HDL metabolism, it is vital to examine the effects of APOE on AD risk and its relationship to aerobic fitness-induced increases in HDL-C. Given potential multiple ways in which exercise may improve cognitive performance and therefore reduce AD risk and the relatively large aerobic fitness-related effect size reported in many studies, clinical trials are needed to determine the effect of aerobic exercise-training on cognitive function in patients with mild AD, notwithstanding the recent NIA consensus statement on general lack of progress.

The demonstration of training-related improvements in neurocognitive function and regional cerebral glucose utilization independent of or interactively with APOE gene would provide momentum for a large-scale clinical trial. A concomitant improvements in HDL-C and inflammatory markers will significantly advance knowledge of the mechanism by which aerobic fitness affects neurocognitive function. A study with the advantage of an experimental design, the use of a control group, and ability to examine the contribution of putative CVD risk factors to AD development and progression is highly desirable. In addition to informing the mechanism by which aerobic fitness can enhance neurocognitive vitality in humans in a subsequent large-scale clinical trial, it will help quantify the effects of aerobic fitness on biomarkers, neurodegeneration, and brain glucose homeostasis. For populations such as AAs with disproportionately higher rates of CVD risk and pathology, a confirmatory large scale trial will validate the role of aerobic fitness as an adjunct treatment to ameliorate the physical, psychological, and economic burden associated with AD at individual levels. In addition to providing evidence leading to a scientific basis for a change in health policy and standard of care, society is also likely benefit from reduction in the economic burden.

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*Review Article*

# Hormone Replacement Therapy and Risk for Neurodegenerative Diseases

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Over the past two decades, there has been a significant amount of research investigating the risks and benefits of hormone replacement therapy (HRT) with regards to neurodegenerative disease. Here, we review basic science studies, randomized clinical trials, and epidemiological studies, and discuss the putative neuroprotective effects of HRT in the context of Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and HIV-associated neurocognitive disorder. Findings to date suggest a reduced risk of Alzheimer's disease and improved cognitive functioning of postmenopausal women who use 17$\beta$-estradiol. With regards to Parkinson's disease, there is consistent evidence from basic science studies for a neuroprotective effect of 17$\beta$-estradiol; however, results of clinical and epidemiological studies are inconclusive at this time, and there is a paucity of research examining the association between HRT and Parkinson's-related neurocognitive impairment. Even less understood are the effects of HRT on risk for frontotemporal dementia and HIV-associated neurocognitive disorder. Limits to the existing research are discussed, along with proposed future directions for the investigation of HRT and neurodegenerative diseases.

## 1. Introduction

Hormone replacement therapy (HRT), defined here as use of various types of estrogen alone or in conjunction with progestins (synthetic or exogenous progestogen), has long been studied as a possible prophylactic against Alzheimer's disease. While the association between HRT and Alzheimer's disease has been explored through several observational and randomized clinical trials to date, the relationship between HRT and other neurodegenerative diseases has received relatively little attention. In this review, we explore the body of research on HRT as a prophylactic against various neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and HIV-associated neurocognitive disorder. In reviewing observational studies, randomized clinical trials, and basic science studies, we find evidence that some forms of HRT are neuroprotective, resulting in preservation of cognitive abilities in healthy postmenopausal women, improvement of Parkinson's symptoms, and variably altering risk of neurodegenerative disease.

## 2. Alzheimer's Disease

Alzheimer's disease (AD) represents the most common neurodegenerative disease, accounting for more than 50% of all dementia types [1]. Within the United States alone, national prevalence estimates indicate that AD affects 2.4 million individuals aged 70 and older [1, 2]. With increasing age, AD progressively affects more individuals, affecting 2.5% of those aged 71–79, 18% of those aged 80–89, and 30% of those aged 90 and older [1, 2].

Cognitive decline in AD is characterized by insidious onset and gradual progression over a course of several years [3–5]. Clinical research has identified subtle losses

of cognitive functioning that precede AD. Studies have consistently shown that a deficit in episodic or verbal memory, specifically the ability to encode novel information, is an early symptom of AD and often presents several years before a formal diagnosis of AD [3, 6–9]. Such observations have led to the identification of a preclinical stage of AD that represents the transition between normal aging and AD. Specifically, mild cognitive impairment (MCI) represents the mild neurocognitive decline that occurs in the presence of relatively intact day-to-day functioning [4, 5]. Although there are several subtypes of MCI, the subtypes that are at increased risk for the development of AD involve predominant memory impairment. It has been estimated that approximately 10–15% of those diagnosed with MCI with predominant memory impairment convert to AD per year [4, 5]. The identification of MCI as a possible prodrome to AD, as well as the recent development classifier algorithms that assess later risk for AD based on a variety of clinical factors [10], leaves open the potential for initiating therapies, including HRT, that may prevent progression to AD in those at risk.

*2.1. Estrogen and Risk for AD—Observational Studies.* Incidence rates indicate the risk of AD among women is double that of men after the age of 80, even after controlling for protective factors such as education [11]. The higher incidence rate of AD among women have led to explorations on the association between estrogen deficiency and AD.

Observational studies have examined both HRT and estrogen replacement therapy (ERT), or estrogen alone, in relation to incidence of AD (see Table 1). For instance, in a sample of 514 women enrolled in the Baltimore Longitudinal Study of Aging, Kawas et al. found that ERT was associated with significantly reduced risk for AD [12]. Although the duration of use ranged between 1–15 years, the data did not show a significant effect for duration of ERT. In addition, no effect was observed for age of menopause. In another observational study reported by Tang et al., ERT was also associated with significantly reduced risk for AD in a sample of 1124 women enrolled in the Manhattan Study of Aging [13]. Here, however, an inverse relationship was observed for duration of use and risk for AD, with the lowest risk noted for women taking estrogen for longer than one year. Other observational studies have provided moderate support for decreased AD risk with ERT and the importance of duration of use. Using retrospective data on a sample of 355 women, Paganini-Hill and Henderson found that ERT was associated with moderately reduced risk for development of AD [14]. An inverse relationship was seen for duration of ERT and risk for all-cause dementia (AD as well as other causes of dementia), with those on ERT for seven or more years having the lowest risk for AD. While findings from these observational studies suggest that ERT may reduce risk of AD, given the nature of observational studies the findings may be affected by several biases. Specifically, the women who decided to take ERT for several years may have been healthier to begin with; they may have also been more proactive in seeking early postmenopausal treatment due to higher education and/or availability of resources. An additional criticism is the lack of controls in the studies; for instance, all observational studies described above involved varied ERT regimens among all participants rather than a uniform ERT regimen. Thus, the findings of the observational studies present with several limitations.

Although all of the studies examined above have included women who underwent natural menopause, recent observational studies have examined the differences between women who underwent natural versus surgical menopause [15]. In one, women who had surgical menopause demonstrated an increased long-term risk for cognitive impairment compared to women with natural menopause. In another paper based on the same data, the same researchers reported a linear trend, with increased risk seen with younger age at oophorectomy (bilateral or unilateral) [16]. These findings suggest that earlier age of surgical menopause increased risk of cognitive impairment and that estrogen deficiency may initiate risk for neurological diseases such as AD. Notably, the researchers also found increased risk of depression and cardiovascular disease among women with history of bilateral oopherectomy, suggesting that the relationship between surgical menopause and cognitive impairment may be multifactorial [17, 38].

*2.2. Randomized Clinical Trials of HRT in Healthy and At-Risk Women.* While observational studies generally support a neuroprotective role for ERT against AD, the results of randomized clinical trials (RCTs) have been equivocal. To date, the largest study has been the Women's Health Initiative Memory Study (WHIMS), an ancillary study of the Women's Health Initiative (WHI), a prospective study that enrolled 7479 postmenopausal women [39, 40]. A total of 4532 women with natural menopause (intact uterus) were randomized into a trial comparing conjugated equine estrogen (CEE) + medroxyprogesterone (MPA) versus placebo [40]. However, the trial was discontinued before completion due to unexpected health risks. Despite the early termination, data revealed that women who received CEE + MPA demonstrated greater cognitive decline compared to the placebo group [40]. Additional analyses revealed that risk for dementia was doubled for women who received CEE + MPA compared to the placebo group [39]. Taken together, data from the WHIMS demonstrated a higher incidence of dementia and greater cognitive decline among hormone users relative to placebo groups.

Although the WHIMS has been considered one of the largest and longest randomized studies examining HRT and cognitive deficits, generalizability of the findings is affected by several limitations. First, external validity of the WHIMS findings has come into question, as the participants in the treatment group were at high risk for cardiovascular and cerebrovascular disease; thus the higher rates of dementia may have been attributed to vascular disease. Second, in their analyses of the WHIMS data, the researchers included all dementia types into an "all-cause" dementia that included AD, vascular dementia, dementia due to Parkinson's disease, and frontotemporal dementia, thus limiting the interpretation of results. Third, a methodological limitation included the unavailability of baseline cognitive measures prior to

Table 1: Observational studies of ERT and risk for dementia.

| Study (reference) | Sample description | Overall findings |
|---|---|---|
| Paganini-Hill and Henderson [14] | 355 postmenopausal women (165 users; 190 nonusers) with a mean age of 86.5 years at death; retrospective data from the Leisure World, Laguna Hills cohort | ERT (not specified) for 1–7 years was associated with reduced risk for AD (OR: 0.67, CI 95% 0.38–1.17) compared to nonusers. Risk for AD decreased with longer duration of use. |
| Tang et al. [13] | 1124 healthy postmenopausal women (156 users; 968 nonusers), with a mean age of 74.2, enrolled in the Manhattan Study of Aging | After controlling for age, education, and ethnicity, ERT (majority used CEE) for 6–8 years was associated with lower risk for AD (OR 0.50, 95% CI, 0.25–0.90) compared to nonusers. Risk for AD decreased with longer duration of use. |
| Kawas et al. [12] | 514 healthy postmenopausal women (230-users; 242-non-users), with a mean age of 65.5, enrolled in the Baltimore Longitudinal Study of Aging | After controlling for education, ERT (not specified) for 1–10 years was associated with lower risk for AD (OR: 0.46, 95% CI, 0.21–0.99) compared to non-users. No effect was observed for duration of use. |
| Rocca et al., [15–17] | 813 women with unilateral oophorectomy, 676 women with bilateral oophorectomy, and 1,472 women who did not undergo oophorectomy. | Women who underwent oophorectomy (unilateral or bilateral) before onset of menopause were at increased risk for cognitive impairment or dementia (OR: 1.46, 95% CI, 1.13–1.90) compared to women who did not undergo oophorectomy. Risk increased with younger age at oophorectomy. |

treatment; thus, participants may have already been cognitively impaired prior to beginning HRT. Still another criticism has been the age of the participants; participants were age 65 or older, at least a decade past the average age of menopause. Together, these limitations have called into question the validity of the WHIMS findings, suggesting that the WHIMS may not be the best model for understanding the effect of HRT on Alzheimer's disease.

Another limitation in the generalizability of the WHIMS involves the type of HRT that was used. Specifically, it has been pointed out that CEE does not contain the hormone 17$\beta$-estradiol, [41] the estrogen compound that has been shown in basic science studies to be neuroprotective [42–44]. In addition, the greater rates of dementia seen among participants of the CEE + MPA study trial of the WHIMS suggest that simultaneous use of MPA may present additional risk [45]. Indeed, consistent with WHIMS findings, a recent randomized-controlled study by Maki et al. found that women receiving CEE + MPA for four months demonstrated mild declines in verbal memory compared to women receiving placebo [21]. Additionally, a recent comparison of several different HRT types has provided some insight into which treatment provides the most cognitive benefit. Using functional neuroimaging as an outcome measure, Silverman et al. compared the cerebral metabolic activity associated with three hormone regimens over the course of one year: 17$\beta$-estradiol (E2), CEE, and CEE + progestin [24]. Results revealed that the E2 group performed significantly better on verbal memory than the CEE group. This group also demonstrated higher metabolism in the receptive language and auditory association areas. Additionally, the CEE + progestin group demonstrated lower metabolism in areas associated with long-term memory storage (i.e., mesial and inferior lateral temporal regions) compared to the CEE group. Taken together, these findings suggest that E2-based therapies may provide the most beneficial neuroprotective effect. In addition, the Silverman et al. study suggests that combination therapies that include progestin may actually dampen the beneficial effects of estrogen.

Since the discontinuation of the WHIMS trials, the case for ERT in reducing the risk for AD and improving the cognitive functioning of postmenopausal women has continued to gain at least modest support through further RCTs. Indeed, results from several RCTs published in the past few years have demonstrated support that E2 formulations are associated with a reduced amount of decline in verbal memory among healthy postmenopausal women when compared to controls. The benefits of these treatments have been observed in trials with durations ranging from three months to two years (see Table 2) [18, 22–24]. In contrast, at least one study has found no benefit on verbal memory associated with E2 compared to placebo [20]; however, it was noted that the women in that study used E2 for only two months. Thus, it is possible that the effects of E2 on verbal memory may be evident only after three months or more. In a separate study, Joffe et al. found that E2 was not associated with an improvement in verbal memory scores but rather decreased likelihood for errors during the memory tasks [19]. Specifically, women on E2 demonstrated less perseverative errors during recall tasks compared to women on placebo. These women, as a group, were also less likely to demonstrate an interference effect when retaining previously learned information. Thus, although E2 was not found to enhance verbal memory scores per se, the authors concluded that E2 enhanced verbal information processing by decreasing the forgetfulness of a response already given [19].

*2.3. Neuropathological and Neurophysiological Studies of HRT: Relevance to AD.* While results of recent RCTs show modest support for a beneficial effect, evidence from histopathological and neurophysiological studies has provided stronger support for estrogen's neuroprotective effects, particularly for the neurodegenerative disease process thought to underlie AD [46–48]. Neuroimaging and autopsy results have

Table 2: Randomized clinical trials of HRT and verbal memory.

| Study (reference) | Hormone treatment used | Sample size | Age | Outcome measure | Overall findings |
|---|---|---|---|---|---|
| Bagger et al., [18] | E2 2 mg + varied progestins versus placebo for 2 years | 261 | 54.1 | Cognitive screening task | Followup study of women randomized 5, 10 and 15 years earlier to HRT or placebo during clinical trials. Logistic regression showed that for women who received HRT for 2-3 years, the relative risk for cognitive impairment was significant decreased by 64% compared to the never users. Long-term/current users of HT also demonstrated a decreased risk of 66% compared to the never users. |
| Joffe et al. [19] | E2 0.5 mg versus placebo for 12 months | 52 | 40–60 | Verbal memory; Functional MRI | Women on E2 had fewer perseverative errors during verbal recall when placebo-treated women. Women on E2 also showed greater retention of new information without interference. |
| LeBlanc et al., [20] | Estradiol 2 mg versus placebo for 2 months | 32 | 53.26 (treatment) 52.08 (placebo) | Verbal memory | Women on estrogen therapy did not show higher cognitive performance on verbal memory tasks compared to women on placebo. |
| Maki et al., [21] | (CEE) + medroxyprogesterone acetate (MPA) versus placebo for 4 months | 158 | 51.9 (treatment) 52.4 (placebo) | Verbal memory | Modest negative effects on verbal memory (short- and long-term recall) were found in the HRT versus placebo group. |
| Dumas et al. [22] | E2 2 mg versus placebo for 3 months | 22 | 50-62 (younger) 70–81 (older) | Verbal memory | All women were administered the antimuscarinic drug scopaline (SCOP) or placebo. E2 pretreatment significantly decreased the anticholinergic drug-induced impairments on verbal memory task for the younger group only compared to the older group. |
| Tierney et al. [23] | E2 1 mg versus placebo for 2 years | 142 | 61–87 | Verbal memory | Women on E2 who scored at or above average showed less decline in delay verbal memory compared to women on placebo. |
| Silverman et al. [24] | 17$\beta$-estradiol (E2) versus conjugated equine estrogen (CEE) versus CEE + P for 1 year | 53 | 50–65 | Verbal memory; FDG-PET | Women on E2 had significantly higher verbal memory than CEE and showed higher metabolism in Wernicke's and auditory association. E2 was also associated with higher metabolism in mesial and inferior lateral temporal regions and inferior frontal cortex compared to PE. |

indicated that $\beta$-amyloid and tau proteins are involved in the structural changes that lead to AD pathology, particularly in the hippocampus and other medial temporal regions, as well as the parietal and frontal cortical regions [49]. Evidence has shown that estrogen (particularly E2) provides protection against $\beta$-amyloid-induced damage and tau-related changes [50]. Observational and RCT studies that also utilized neuroimaging outcomes have also been supportive of the benefits of 17$\beta$-estradiol, particularly in the brain regions that show preclinical abnormalities in individuals who are at risk for AD. For instance, as mentioned earlier, E2 has been associated with higher metabolism in language processing and auditory association areas compared to other HRT regimens (CEE or CEE + MPA) [24]. However, observational studies and RCTs have also demonstrated support for varied ERT regimens. Compared to nonusers, long-term ERT (E2 or CEE for an average of 15 years) has been associated with increased cerebral blood flow to the hippocampus and left superior temporal gyrus at a two-year followup [51]. Further, compared to placebo, a four-month trial of ethinylestradiol and progestin was associated with increased activation in brain regions associated with the left middle/superior frontal cortex, and left inferior parietal cortex during verbal memory encoding tasks on functional magnetic resonance imaging [52]. Finally, in another study, long-term users of ERT (E2 or CEE for an average of 18 years) demonstrated higher

density of muscarinic receptors in the hippocampus and prefrontal cortex than individuals who had never used ERT, suggesting that one of the neuroprotective effects of E2 or other ERT regimens could also include the maintenance of the cholinergic system in the hippocampus and frontal cortex [48].

A recently proposed explanation may explain the inconsistent results of the aforementioned observational studies and RCTs. Known as the "healthy-cell bias" [53], the hypothesis is that E2 may selectively benefit healthy neurons. In the context of human studies, based on the findings from observational studies and RCTs, this hypothesis predicts that E2 can be protective if initiated before or during times of neuronal stress, but harmful if given after the cells have progressed toward degeneration. In their study, Chen et al. administered E2 to rat hippocampal neurons exposed to $\beta$-amyloid, using varied doses and dose schedules (acute versus continuous versus intermittent). Data indicated that neurodegeneration was prevented when E2 was administered before or during $\beta$-amyloid exposure, and a continuous dose was found to demonstrate the strongest effects. In contrast, exposure to higher doses of E2 actually worsened neuronal death when $\beta$-amyloid was present. Additionally, E2 administered after $\beta$-amyloid exposure exacerbated neuronal death. It was concluded that the best E2 dosing was pretreatment and continuous exposure to prevent degeneration. Consistent with the "healthy-cell bias" hypothesis, Dumas et al. demonstrated a selective benefit of 17$\beta$-estradiol toward cognitively intact women [22]. A group of 142 postmenopausal women (age range: 61–87) were randomized to receive E2 ($n = 70$) or placebo ($n = 72$) for two years. Verbal memory was assessed at baseline and at 1-year and 2-year followup. Results revealed that women who received E2 and who performed at or above average on verbal recall at baseline demonstrated higher scores at the 1-year and 2-year followup compared to the placebo group. In contrast, women who received E2 and performed below average on verbal recall at baseline showed no significant difference compared to the placebo group. Dumas et al. concluded that these findings provided support to the healthy cell bias hypothesis, as they considered it improbable that women with a normal score or better had significant neurodegenerative changes [22]. Notably, the women who benefitted from estrogen exposure were age 70 (average) and approximately 20 years postmenopause, suggesting that older women who have intact verbal memory can benefit from a new regimen of ERT late in life, as long as they have not demonstrated memory impairment. Interestingly, basic science research has supported the biased neuroprotective effect of E2 toward healthy individuals; in fact, the presence of apoplipoprotein E4 (APOE4) genotype has been found to reduce the neuroprotective role of E2 in an animal model [54]. Thus, an alternative explanation for the findings of Dumas et al. could be that the women who demonstrated lower than average recall at baseline may have had the APOE4 genotype; in turn, they may have not experienced the neuroprotective effects of ERT. The healthy cell bias hypothesis also helps explain the finding, reported in most observational studies, of an inverse relationship between length of HRT treatment and risk for AD.

Other investigators have hypothesized that there may be a "critical period" for postmenopausal women during which 17$\beta$-estradiol selectively provides a beneficial effect for younger as opposed to older women with an intact uterus [41, 55]. This hypothesis has also received support from at least one RCT. For example, LeBlanc et al. randomized 22 postmenopausal women to receive either E2 or placebo for 3 months [20]. At the end of the trial, the antimuscarinic drug scopolamine (SCOP) was administered before a verbal task to initiate anticholinergic-induced memory impairment. Results showed that E2 pretreatment significantly decreased the anticholinergic-induced impairment on the verbal memory task for the younger group (age 50–62); however, the benefit of E2 was not observed in the older group (age 70–81). Interestingly, the beneficial effects of E2 were only observed during the anticholinergic challenge with SCOP and not during the placebo challenge. LeBlanc et al. concluded that younger women benefit from E2 more than older women, and that the benefits of E2 in younger women may be observed only when the cholinergic system is temporarily disrupted. Consistent with this finding is that younger women have a higher density of muscarinic receptors than older women, and thus may be more sensitive to cholinergic changes [48]. Thus, it is plausible that the women in the aforementioned WHIMS may have been past the "critical period" for the beneficial effects of E2.

*2.4. Summary—AD.* Taken together, the findings from studies employing a variety of methods demonstrate that some forms of ERT are neuroprotective, resulting in preservation of cognitive abilities and reduced risk of AD. While some studies have affirmed that young and healthy postmenopausal women may benefit the most from estrogen exposure, other studies have suggested that older and healthy women with intact verbal memory can also benefit from estrogen. The consistent findings from the observational studies reviewed above seem to be that ERT (most commonly CEE), with a minimal duration of at least one year, is beneficial in reducing risk for AD among healthy postmenopausal women. Although benefits have been observed among varied regimens (CEE, CEE + P, E2) [48, 51, 52]; the most beneficial estrogen formulation seems to be E2 unopposed by progestin [24, 50]. Randomized clinical trials in healthy, postmenopausal women have suggested that E2 has been most beneficial in reducing cognitive decline, particularly verbal memory, which is the predominant symptom of early AD [18, 22–24]. Additionally, both observational and RCT studies utilizing neuroimaging outcomes have been supportive of the benefits of E2, particularly in the brain regions that show preclinical abnormalities in individuals who are at risk for AD [21, 24, 51, 52].

## 3. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD, with an estimated

prevalence of 0.3% in the general population. Risk increases with age, with a prevalence of 1% in those over 60, and 4% in those 80 years and older [56]. Many, but not all, studies have reported higher risk for PD and younger age of onset in males [57–66]. This observation, along with the fact that the neuropathological process underlying PD commonly begins before menopause, suggests that estrogen may play a modulatory role. In addition, estrogen has a direct modulatory affects on dopaminergic functioning [67]. Together, these observations suggest a potential protective effect of estrogen against PD, or ameliorative impact on symptoms.

*3.1. Estrogen and PD Symptoms.* A variety of studies have addressed the impact of estrogen on PD. Perhaps the most indirect are observational studies of PD symptoms during the menstrual cycle. Early studies in the 1980s reported that some female patients with PD had fluctuations in motor symptoms that paralleled presumed fluctuations in endogenous estrogen levels [68, 69], with presumably lower levels of estrogen associated with greater motor symptoms. However, more recent studies have shown mixed results. Kompoliti et al. did not find significant correlation between endogenous hormone levels and motor examination in the "off" state (a state of decreased mobility as a result of nonresponsiveness to medication) among female PD patients examined at various times during their menstrual cycle [70].

A small number of prospective studies of ERT and PD have also been reported, with mixed results (Table 4). Strijks et al. did not find a significant dopaminergic effect in their 8-week placebo-controlled, randomized, double-blind trial pilot study of E2 in 12 postmenopausal female patients under the age of 80 [35]. However, an 8-week double-blind, parallel-group, prospective study using Premarin (CEE) versus placebo in PD patients with motor fluctuations showed a statistically significant improvement in "off" times (i.e., when dopamine agonist medications have diminished efficacy) among the estrogen treated group [36]. Further, another double-blind, placebo-controlled crossover study of high-dose transdermal E2 in 8 postmenopausal women with mild-to-moderate PD demonstrated a slight anti-Parkinsonian effect without significantly worsening dyskinesias [34].

Although the overall symptomatic effect of ERT on PD remains unclear, these early studies raised the possibility that some forms of estrogen may mitigate the symptoms of PD. Despite this early optimism, a more recent multicenter, randomized, double-blind, placebo-controlled, pilot trial of CEE in postmenopausal women with PD experiencing motor fluctuations did not find any benefit of ERT in ameliorating symptoms [37]. In that study, 23 women received either 0.625 mg/day of CEE or matching placebo for 8 weeks. None of the outcome measures, including changes from baseline to study completion in Unified PD Rating Scale scores, "on" time (i.e., duration that dopamine agonist medication is effective), dyskinesia ratings, and results from neuropsychological testing, were significantly different between the placebo and treatment groups, although the authors emphasized a nonsignificant trend of improvement on the total and motor scores of the Unified PD Rating Scale. It is conceivable that the null findings were due to the small sample size; however, the existing literature on ERT and PD symptoms remains equivocal at this time.

*3.2. HRT and Observational Studies of PD Risk.* Epidemiological studies of the protective effects of HRT against PD have been mixed as well (Table 3). The relationship between lifetime reproductive events and PD was examined by Martignoni et al. Comparing a large sample of women diagnosed with PD to healthy controls, they found that the duration of reproductive life was similar between the two groups [28]. Time and mode of menopause onset were also similar between the groups; however, women with PD reported less access to HRT. In addition, the PD group overall reported more premenstrual symptoms, fewer deliveries and abortions, and less use of contraception, indicating a relationship between PD and reproductive events. Benedetti et al. reported a case-control study in which women with PD had an earlier reported age of menopause, a higher frequency of hysterectomies, and lower occurrence of HRT [27]. Further, Currie et al. found that ERT in postmenopausal women was associated with a significantly reduced risk of developing PD [29], and Ragonese et al. found that factors reducing estrogen stimulation during life were associated with development of PD [30]. Specifically, PD was significantly associated with shorter fertile life lengths (<36 years) and a longer cumulative length of pregnancies (>30 months). This group later reported a significant correlation between age of PD onset and both age at menopause and fertile life duration [32]. Despite these findings, others have found contrary results. Popat et al. found that the association of postmenopausal HRT and PD risk depended on the type of menopause [31]. Among women with history of hysterectomy (with or without an oopherectomy), ERT use was associated with a 2.6-fold increased risk for PD, and a trend for additional risk was noted for increasing duration of estrogen use. Conversely, among women with natural menopause, no increased risk of PD was observed with HRT (ERT alone or in conjunction with progestin). Contrary to the findings of Benedetti et al., earlier age of menopause was associated with reduced risk of PD. Further, Simon et al. recently reported results of a 22-year prospective study of 244 participants in the Nurses' Health Study who developed PD [33]. Among their sample, risk of PD was not significantly associated with reproductive factors or HRT use. However, they did find that use of HRT may modify the associations of smoking and caffeine with PD risk; specifically, the inverse relationship between caffeine use and risk of PD was observed only in non-HRT users. Further, whereas the researchers also reported an inverse relationship between pack-years of smoking and risk of PD for both HRT users and nonusers, risk was reduced more in the latter group. As such, HRT use appeared to attenuate the observed beneficial effects of caffeine use and tobacco smoking. Of note, this study did not separately analyze the data based on type of HRT.

Table 3: Case-control and epidemiological studies of HRT and Parkinson's disease.

| Study (reference) | Sample description | Overall findings |
|---|---|---|
| Marder et al. [25] | 87 women with Parkinson's disease without dementia (PDND), 80 women with Parkinson's disease with dementia (PDD), and 989 nondemented healthy women. | ERT reduced risk of dementia among the PD-only sample (OR = 0.22, 95% CI: 0.05–1.0), and also when PDD patients were compared to healthy controls (OR = 0.24, 95% CI: 0.07–0.78). ERT did not affect the risk of PD. |
| Fernandez and Lapane [26] | Data from 10,145 elderly women with PD available via the Systematic Assessment in Geriatric drug use via Epidemiology (SAGE) database. Included 195 women with PD who received estrogen and 9950 who did not receive estrogen. | Independent of age, estrogen users had better cognitive functioning and were more independent with regards to activities of daily living. More estrogen users were depressed and likely to be taking antidepressant medications. |
| Benedetti et al. [27] | 72 women with PD and 72 healthy women. | The PD group had undergone hysterectomy (with or without unilateral oophorectomy) more than the control group (OR = 3.36; 95% CI: 1.05–10.77). The PD group had more frequent occurrence of early menopause (< or = 46 years) (OR = 2.18; 95% CI: 0.88–5.39). The PD group used ERT for at least 6 months after menopause less frequently than the control group (14%; OR = 0.47; 95% CI = 0.12–1.85). The PD group did not have earlier menopause than the control group. |
| Martignoni et al. [28] | 150 women with idiopathic PD and 300 healthy women, all postmenopausal. | Duration of reproductive life was similar between women with PD and those without PD. Women with PD reported less access to HRT. The PD group also reported more premenstrual symptoms, fewer deliveries and abortions, and less use of contraception, indicating a relationship between PD and reproductive events |
| Currie et al. [29] | 68 women with PD and 72 healthy women, all postmenopausal. | 50% of women in the control group took ERT, as compared to 25% of women in the PD group. Women who had taken postmenopausal ERT were less likely to develop PD than those who had not (odds ratio, 0.40; 95% CI: 0.19–0.84). Among women with PD, postmenopausal ERT was not associated with age of onset. |
| Ragonese et al. [30] | 131 women with idiopathic PD and 131 healthy women. | PD was significantly associated with a fertile life length of less than 36 years (OR 2.07; 95% CI: 1.00 to 4.30). PD was also associated with a cumulative pregnancy length of longer than 30 months (OR 2.19; 95% CI: 1.22 to 3.91). There was an inverse association between PD and surgical menopause (OR 0.30; 95% CI: 0.13 to 0.77). |
| Popat et al. [31] | 178 women with PD and 189 healthy women. | Among women with history of hysterectomy (with or without an oopherectomy), ERT use was associated with a 2.6-fold increased risk for PD, and a trend for additional risk was noted for increasing duration of estrogen use. Among women with natural menopause, no increased risk of PD was observed with HRT (ERT alone or in conjunction with progestin). Earlier age of menopause was associated with reduced risk of PD. |
| Ragonese et al. [32] | 145 women with PD. | A significant correlation was found between age at PD onset and age at menopause, and also between age at PD onset and fertile life duration. |
| Rocca et al. [16, 17] | 1,252 women with unilateral and 1,075 women with bilateral ophorectomy, and 2,368 referent women. | Women who underwent either unilateral or bilateral oophorectomy had an increased risk of parkinsonism compared to referent women (HR 1.68; 95% CI: 1.06–2.67). This risk increased with younger age at oophorectomy. |
| Simon et al. [33] | 22-year prospective study of 244 women with PD enrolled in the Nurses' Health Study. | Risk of PD was not significantly associated with reproductive factors or HRT. The association of smoking and caffeine with PD risk was modified by HRT, however. Based on a very small sample (4), women using progestin only hormones had increased risk for PD. |

Table 4: RCTs of ERT and Parkinson's disease.

| Study (reference) | Hormone treatment used | Sample Size | Outcome measure | Overall findings |
|---|---|---|---|---|
| Blanchet [34] | High-dose transdermal E2. Cross-over design with 2 weeks on E2, 2 week washout, and 2 weeks on placebo | 8 | Therapeutic threshold for levodopa. | All but one participant had levodopa-induced dyskinesia at start of study. After 10 days of E2 treatment a significant reduction was observed in the anti-parkinsonian threshold dose of intravenous levodopa without significantly worsening dyskinesias |
| Strijks et al. [35] | 17$\beta$-estradiol (E2) versus placebo for 8 weeks | 12 | Motor score from the Unified Parkinson's Disease Rating Scale (UPDRS); patient report of subjective changes. | No differences in outcome measures between E2 and placebo. |
| Tsang et al. [36] | CEE versus placebo for 8 weeks | 40 | UPDRS, timed tapping score, Hamilton Depression Scale, patient self-report. | "On" and "off" times, and motor score on the UPDRS improved with estrogen. |
| The Parkinson Study Group Poetry I Investigators [37] | CEE versus Placebo for 8 weeks | 23 | Primary outcome was ability to complete the trial. Other outcome measures included adverse events, UPDRS, "on" time, dyskinesia ratings, and neuropsychological functioning | The estrogen group showed a trend for improvement on the total and motor UPDRS scores. |

In one of the largest observational studies to date, Rocca et al. examined 1,252 women with unilateral oophorectomy, 1,075 women with bilateral oophorectomy, and 2,368 controls for development of PD. Data for the participants were collected until death or the termination of the study using direct or proxy interviews, neurologic examinations, medical records, and/or death certificates. The authors found that women who underwent either unilateral or bilateral oopherectomy before the onset of natural menopause, thereby decreasing endogenous estrogen levels, had an increased risk of parkinsonism compared with referent women. Further, risk increased with younger age at oophorectomy. The findings were similar regardless of unilateral or bilateral oopherectomy. Importantly, while the authors reported a trend, the surgical menopause group was not at increased risk for PD.

Although these studies might appear to provide conflicting results, complex factors are at play. The indication for HRT (posthysterectomy, posthysterectomy + oopherectomy, natural menopause), the specific type of HRT (CEE, E2, estrogen/progestin combinations), and other variables may combine in ways yet unknown to increase or decrease PD risk. Clearly, further study is necessary.

*3.3. Studies of HRT and Dementia due to PD.* PD is also associated with cognitive decline, with anywhere between 24–31% becoming demented [71]. PD dementia is considered a subcortical dementia, with associated deficits ranging from simple motor ability to higher-order cognitive functions [72]. Despite the high incidence of neurocognitive dysfunction in PD, the relationship between HRT and dementia in those with Parkinson's disease has received considerably less attention. Only two case-control studies were found. Marder and colleagues investigated risk of PD both with and without dementia among a sample of 1156 women. They reported that ERT protected against development of PD-associated dementia, but not against PD itself [25]. Similarly, Fernandez and Lapane found that estrogen use was associated with better cognitive functioning and greater independence in activities of daily living among a large sample of elderly women living in nursing homes [26]. They also noted that estrogen users were more depressed and likely to be on an antidepressant as compared to nonusers. One-year death rates were comparable between estrogen users and nonusers.

*3.4. Mechanisms of Estrogen Action in PD.* While epidemiologic, observational, and experimental studies of ERT and PD have produced equivocal results, the biological mechanisms for a beneficial effect of estrogen upon dopaminergic functioning are less so. There are two general mechanisms of action through which estrogen might influence PD: symptomatic and neuroprotective. Estrogen receptors have been located in the nuclei of nigral dopaminergic (DA) neurons, including estrogen receptor alpha (ER$\alpha$) and beta (ER$\beta$) [73, 74], suggesting that estrogen might therefore directly influence DA functioning. ER$\alpha$ has also been found in midbrain glial cells [75], and ER$\beta$ in striatal medium spiny neurons [74]. Novel surface membrane estrogen receptors have also been described [76, 77]. Perhaps related to these, administration of exogenous conjugated estrogens results in an increase in binding of the DA transporter ligand TRODAT in otherwise healthy postmenopausal women [78]. It has also been shown that, in the absence of nigral neuroprotection, central E2 synthesis limits striatal DA loss caused by 6-OHDA in male rodents, implicating a modulatory effect on DA function [79]. These studies provide evidence that

estrogens may upregulate the nigrostriatal pathway, either pre- or postsynaptically, by an effect on nuclear or surface membrane estrogen receptors.

Estrogen's neuroprotective actions have been well established. In PD, there are animal models that are exquisitely specific for nigral cell death, of which the 6-hydroxydopamine (6-OHDA) and MPTP/MPP+ models are perhaps the best known [80, 81]. There is ample evidence that both endogenous and exogenous estrogen ameliorate DA depletion in the MPTP/MPP+ model [75, 82–91]. There is similar evidence that estrogen is neuroprotective in the 6-OHDA animal model [79, 92–96], a methamphetamine model [97–100], and a wide range of other relevant animal models [101–103]. The exact mechanisms of neuroprotection, however, are not clear. Studies have shown a role for binding of estrogen to the nuclear estrogen receptor [104], the ER$\alpha$ subtype, [105] ER$\alpha$ with a glial contribution, [75] ER$\alpha$ + ER$\beta$ [106], and ER-independent mechanisms [88]. This has implications for potential therapeutic agents, as some estrogen analogues lack activity at one or both nuclear receptors; while others, such as the "inactive" enantiomer E2, may have no ER binding activity at all. E2 has been shown in the MPTP model to have neuroprotective properties [101], and has been investigated as a possible neuroprotective agent [107].

It is important, however, to recognize the imperfect nature of these preclinical models. First, while PD is a chronic, slowly progressive disorder, the aforementioned animal models use agents that cause acute toxicity. Second, despite the wide use of these models over the past two decades and the demonstration in preclinical models that many agents are neuroprotective against 6-OHDA, MPTP, or both, none of these agents have proven neuroprotective in human subjects with PD. There may be a simple explanation for this. We now know that neurodegeneration in most cases of familial PD is due to impaired ubiquitin-proteosomal function and alpha-synuclein protein aggregation [108]. Although the relationship between these abnormalities and those replicated by the 6-OHDA and MPTP models are complex, it appears likely that any agent that will be neuroprotective in humans with idiopathic PD will need to act to reduce alpha-synuclein aggregation. This can occur either by reducing its synthesis, reducing protein aggregation, enhancing its elimination, or reducing the toxic effects of excessive alpha-synuclein. Only recently has evidence been found that estrogen has the ability to act on alpha-synuclein in a beneficial manner. Hirohata et al. found a variety of sex hormones, including estriol, estradiol, estrone, androstenedione, and testosterone to exert significant antiaggregation and fibril-destabilizing effects on alpha-synuclein *in vitro*. Estradiol was especially effective [109]. Further, Marwarha et al. showed that activation of ER$\beta$, in conjunction with inhibition of LXR$\beta$, may reduce progression of PD by slowing $\alpha$-synuclein accumulation.

*3.5. Summary—PD.* While *in vitro* and non-human *in vivo* experiments have consistently demonstrated evidence for estradiol's neuroprotective activity in dopaminergic neurons and animal models of PD, results of clinical and epidemiological studies are inconclusive at this time. Recent findings of estradiol's modulation of alpha-synuclein indicate a specific mechanism through which the hormone may reduce risk for PD and/or mitigate symptoms. Longer clinical trials with specific estrogen compounds (i.e., 17$\beta$-estradiol), as well as biological markers of disease progress (e.g., neuroimaging), will be more likely to definitively determine if ERT is protective against PD or if it can mitigate the disease. With specific regards to PD-associated dementia, only two case-control studies were located, both suggesting that ERT reduces risk of cognitive impairment in women with PD.

## 4. HIV-Associated Neurocognitive Disorder (HAND)

Internationally, an estimated 33 million individuals have HIV/AIDS, [110] and in many areas women comprise the majority of those infected [111]. Aggressive intervention with a regimen of multiple antiretroviral drugs (combined antiretroviral therapy, or cART) has successfully increased lifespan and attenuated some of the most dire neurological effects of HIV infection. However, cART cannot eradicate HIV, and it has attenuated, not eliminated, the most common neurological complication of HIV, or HIV-associated neurocognitive disorder (HAND) [112]. In this section, we discuss what is known about estrogen and HAND from observational studies in humans, studies in animal models, and *in vitro* studies. No relevant human clinical trials of estrogen for HAND have been published.

HAND is a constellation of cognitive impairments caused by HIV infection [112]. Because of the lack of diagnostic biomarkers, HAND remains largely a clinical diagnosis, made when an HIV+ individual experiences neurocognitive decline, sometimes with concomitant deficits in day-to-day functioning, and only after other conditions that might cause this decline have been ruled out. The severity of HAND ranges between mild neurocognitive impairment with no impact on day-to-day functioning to a debilitating HIV-associated dementia [112]. While the incidence of new cases of HAD has declined dramatically [113, 114], the prevalence of milder forms of HAND has actually increased along with the longevity of the cART-treated HIV+ population [113]. This phenomenon has been variously ascribed to several explanations, including the presence of irreparable CNS damage pre-cART [115], the failure of many cART regimens to adequately penetrate and treat the CNS [116], persistent low levels of HIV despite treatment [117], and to persistent CNS inflammation [118], among others. The latter is particularly relevant to the putative therapeutic benefit of estrogen, as it appears that cART does not always reduce and in some cases may increase, the CNS inflammation [119] that is associated with HAND [120]. Estrogen has significant anti-inflammatory and neuroprotective properties [121–123] and can potentially counteract inflammation in the HIV+ brain, as discussed in more detail below.

There are several other important reasons for investigating the use of estrogen as an adjunctive treatment in

HIV and HAND. First, estrogen and other gonadal steroids have significant effects on the course and presentation of HIV disease itself. For example, women are at increased risk for acquiring HIV compared to men, and this vulnerability may be affected by gonadal hormones [124]. Further, in a macaque model of HIV infection, progestogen-based hormonal contraceptives increased the risk of acquiring simian immunodeficiency virus (SIV), increased disease progression, and increased genital shedding of SIV; whereas treatment with estrogen lowered risk of acquiring SIV [125]. Results of natural history studies suggest a gender role in disease progression, possibly due to hormonal differences. For example, women have lower HIV RNA viral loads at seroconversion compared to men [126], and when adjusted for CD4+ count, women have lower viral loads throughout the course of their infection [127]. While one study found a lower risk of clinical progression to AIDS among HIV+ women versus HIV+ men treated with cART [128], others have found no differences in clinical outcome by gender [129]. A possible explanation for such gender disparity, should it turn out to be valid, is estrogen, which decreases HIV replication in peripheral blood mononuclear cells [130]. However, all such studies must be interpreted with caution because of the reported gender differences between HIV+ men and women in socioeconomic status, risk behavior, substance abuse, and access to care [131], which also affect progression to AIDS [132, 133]. With regards to HAND, whether women develop HAND at the same rate as men or if there are different clinical manifestations of HAND in men and women remains a controversial topic. In part, this is because so few studies had sufficient numbers of females to evaluate. A sub-study of the Women's Interagency HIV Study is beginning to address this problem [134].

There is neurobiological reason to expect a reduction of HIV-related neuropathological changes with ERT. Firstly, microglia are the resident immune cells of the CNS, and these cells play an important role in driving inflammation in many neurodegenerative diseases, thus representing an important target for therapy [135]. In HIV infection, microglia can be infected and/or activated; they are major sources of complete HIV virions, individual neurotoxic viral proteins, proinflammatory substances, and other potential mechanisms that drive neurotoxicity, neuroinflammation, oxidative stress, and neurodegeneration. Microglia express endogenous estrogen receptors [136], and treatment with estrogen is anti-inflammatory provided it is administered early in the course of an insult [121, 123]. Secondly, estrogen's anti-inflammatory effects may directly counteract the neuroinflammation caused by HIV proteins. HIV-infected cells can generate both replication-competent virions and excess viral proteins, which are shed or secreted into the extracellular space. The HIV coat protein, *gp120*, is the binding protein for viral entry [137] and acts as an indirect neurotoxin via its effects on microglia, macrophages, and astrocytes, initiating a cascade of events that damage neurons. Estrogen has been reported to have a broad anti-inflammatory effect on microglia [121]. Estrogen reduces the neuroinflammatory responses to gp120 and exerts neuroprotective effects on gp120-exposed neurons, by raising the levels of neurotrophins, decreasing apoptotic factors, and antioxidant properties [138]. Zemlyak et al. reported two different beneficial effects of estrogen in the amelioration of gp120-induced toxicity: a major effect of attenuating the neurotoxicity of factors released by gp120-treated microglial cultures, and a minor effect of enhancing the ability of neuronal cultures to survive exposure to neurotoxic factors [122]. Another neurotoxic HIV protein, *tat*, the nuclear trans-activating protein, is essential in promoting the transcription and replication of HIV. *tat* can act both directly to harm neurons [139], and indirectly by stimulating macrophages, microglia, and astrocytes to synthesize harmful substances such as proinflammatory cytokines [140], and by increasing free radicals and oxidative stress [141]. In cell culture, 17$\beta$-estradiol suppressed *tat*-activated transcription of HIV in astrocytes [142]. 17$\beta$-estradiol also attenuated the *tat*-induced release of pro-inflammatory mediators in endothelial cells [143], prevented oxidative stress and cell death associated with combined gp120 and *tat* neurotoxicity *in vitro* [144], and prevented gp120/*tat*-induced loss of dopamine transporter function [144].

These observations have led to the proposal that serum estradiol levels be maintained in HIV+ women as a possible neuroprotective agent against HAND [145]. Despite this, there is little clinical information about estrogen and HAND in HIV+ women. A single retrospective study from the pre-cART era, of 84 older (age 40+ years) HIV+ women, reported that hormone replacement therapy (HRT) was associated with a significantly decreased risk of mortality [146]. Of interest, there were six women in the cohort who were diagnosed with HIV-associated dementia, none of whom reported taking HRT. This study has been interpreted by some to indicate a neuroprotective effect of HRT; however, this was not a prospective study that examined cognition in an organized or standardized fashion. However, based on this last report and on the neuroprotective role of estrogen in other inflammatory and degenerative conditions, the role of estrogen and other hormones in HAND has become an area of growing interest among basic scientists.

No studies of HAND or neurocognitive functioning in HIV+ persons have considered hormonal status or use of exogenous hormones. The preponderance of evidence to date indicates that HIV+ men and women develop neurocognitive impairment at a similar rate, when issues such as access to care, education, and substance abuse history are similar. While some have reported a higher occurrence of HIV-associated dementia among women [147], others have not found this [148, 149]. More recently, Martin et al. studied a large well-matched group of adult male and females, stratified by HIV status, all with a history of substance dependence [150]. Participants were abstinent at the time of testing. Whereas the performance of HIV+ men did not differ from HIV-negative counterparts of measures of motor skill and probabilistic learning, the HIV+ women performed worse than their seronegative counterparts, suggesting that women might be more vulnerable to the effects of HIV. However, due to the absence of a nonsubstance-dependent control group, they could not exclude the possibility that the observed differences were due to gender-related differences in the

cognitive effects of addiction. Another study reported no gender difference in rate of neurocognitive decline over time [151]; and still another found that while rates of impairment were similar between men and women, there were some differences in the neurocognitive profiles [148]. Whether this is related to estrogen or other gonadal hormones remains to be determined.

*4.1. Summary—HIV/HAND.* HAND shares many features with other neurodegenerative diseases, including microglial activation and neuroinflammation. Preliminary studies in animal and *in vitro* models indicate that, like many other neurodegenerative diseases, the effect of HIV on the brain may be blunted by treatment with 17$\beta$-estradiol, and possibly other gonadotrophic hormones. This would have to be balanced against the risks of adding estrogen to the regimens of HIV+ patients, both male and female. However, there is a pressing need to determine if HRT may benefit patients with AIDS who remain at risk for HAND even when treated with HAART.

## 5. Frontotemporal Dementia

Frontotemporal dementia, or FTD, is the most common form of a group of related neurodegenerative diseases that primarily affect the frontal and/or temporal lobes. The others include semantic dementia and progressive nonfluent aphasia. Collectively, these have been called frontotemporal lobar degenerative diseases [152], and they are believed to account for an estimated 20% of dementia cases with presenile onset [153].

Only one study to date has addressed the relationship between HRT and FTD. Levine and Hewett reviewed the medical files of all women seen at an Alzheimer's disease center (ADC) in Central California and found that 70% of women diagnosed with FTD had been taking HRT (exact regimen unspecified) when evaluated, as compared to an estimated 24% of the surrounding population [154]. While one easy interpretation would be that women exhibiting cognitive impairment would have been more likely to be placed on HRT before coming to the ADC, only 20% of women diagnosed with AD at the same center had been taking HRT, so it is therefore unlikely that HRT was administered as a result of preclinical cognitive problems. The women diagnosed with FTD were also similar in age to women entering the center with AD (average age of symptom onset was 65, average age of initial evaluation was 70). While poor diagnostic accuracy and estrogen's beneficial effects on mood were cited as possible reasons for the findings, a more compelling reason offered was a marked upregulation of tau in response to E2 administration, as evidenced *in vitro* [155]. The neuropathological correlates of many FTD cases appear to be tau-related, and in some cases directly linked to mutations in the tau gene [156]. In such cases, E2 may increase risk of FTD by increasing production of mutated forms of tau. However, while the role of tau in FTD has been well established, it is now known that it does not account for all forms of FTD [157]. Still the relationship between tau and E2 is a compelling reason to further study the influence of ERT on risk for FTD.

## 6. Summary and Conclusions

In summarizing the evidence discussed above, HRT, in particular ERT, appears to play an efficacious role in treating and preventing several neurodegenerative conditions. Figure 1 depicts putative neurobiological and neurobehaivoral sequelae resulting from 17$\beta$-estradiol use, based on studies reviewed in this paper. The case for a neuroprotective role of HRT and AD is supported by research from epidemiological and RCT studies, which have shown that estrogen, specifically E2 (17$\beta$-estradiol), can reduce the risk for AD and minimize cognitive decline in otherwise healthy women, particularly verbal memory. Based on basic science research, the mechanisms for this neuroprotection may involve E2's protection against $\beta$-amyloid-induced degeneration and may even include the maintenance of the cholinergic system in the hippocampus and frontal cortex. In addition, at least one study has demonstrated that the presence of progestins in combination therapies may actually dampen the beneficial effects of estrogen [24].

Similarly, *in vitro* and non-human *in vivo* experiments have demonstrated E2's neuroprotective effects in dopaminergic neurons and animal models of PD. In addition, E2's modulation of alpha-synuclein indicates a specific mechanism through which the hormone may reduce risk for PD and/or mitigate symptoms. To date, results of clinical and epidemiological studies of ERT alleviating motor symptoms in PD patients have been mixed and warrant further investigation. The effects of HRT on the neurocognitive symptoms of PD have received little attention, with the two case-control studies to date indicating that ERT reduces risk of cognitive impairment in women with PD.

Preliminary studies in animal and *in vitro* models indicate that treatment with E2, and possibly other gonadotrophic hormones, may reduce the effect of HIV on the brain. To date, much research on the neuroprotective effects for HIV neurodegenerative changes has been conducted on animal models and has yet to extend to humans. Nonetheless, preliminary research has suggested that development of HAND may be alleviated by HRT pretreatment. Conversely, and contrary to the findings from other neurodegenerative diseases, there is some evidence that E2 may actually augment risk for FTD via its action on tau.

Additional research is needed to further delineate the molecular mechanisms through which E2 and other estrogens act to delay or prevent neuropathological progression, or possibly cause progression in the case of FTD. Large-scale observational studies that accurately document HRT regimen and control for factors such as depression, education, and medical comorbidities (e.g., vascular risk factors) will also help to elucidate the role of ERT in the neurodegenerative disease etiology. While observational studies and RCTs examining ERT and AD have demonstrated long-term beneficial effects of varied ERT regimens (E2 or CEE), future studies may include long-term followup (5–10 years) of E2-based therapies alone on cognitive measures

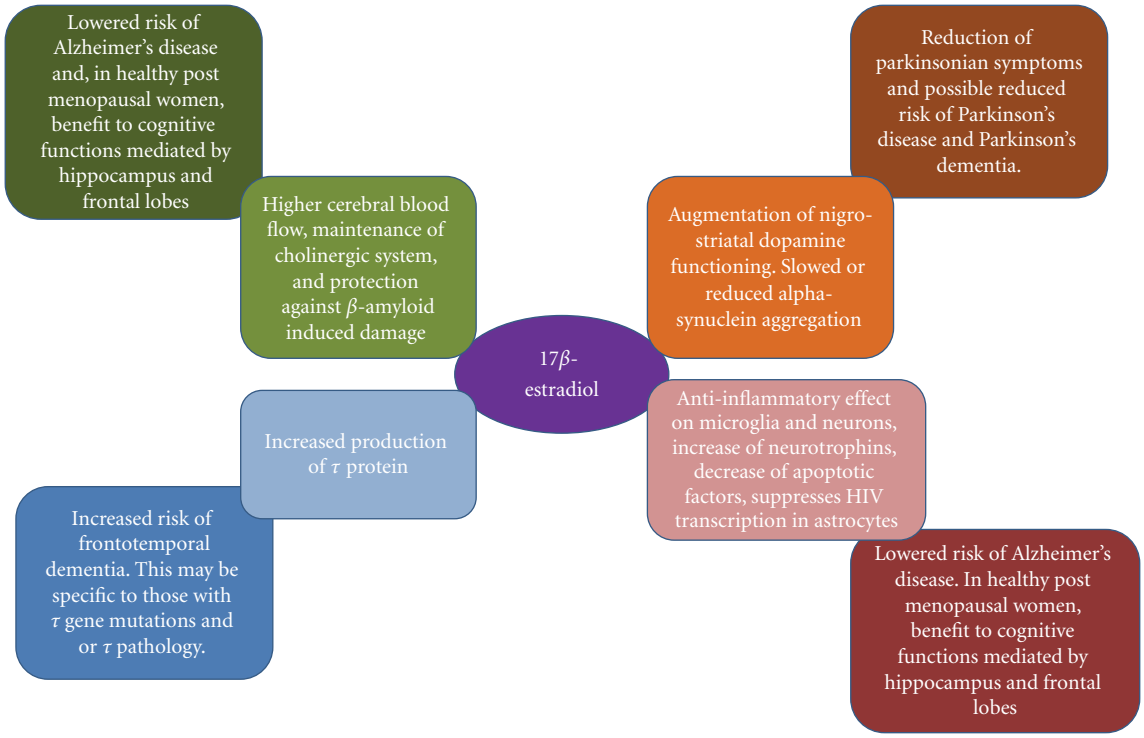


Figure 1: Putative mechanisms through which 17$\beta$-estradiol exerts neuroprotective and neuro-adverse effects. In the context of Alzheimer's disease, Parkinson's disease, and HIV, 17$\beta$-estradiol appears to be neuroprotective. However, frontotemporal dementia is often the result of mutated tau protein and/or tau-related pathology. Because 17$\beta$-estradiol increases production of tau, it may accelerate risk for some forms of frontotemporal dementia.

and neuroimaging outcomes, as such would provide helpful information on the duration of the benefits of E2 following discontinuation.

Notably, possible medical risks should be considered in study of HRT and neurocognitive functioning [45]. For instance, breast cancer is often a substantial concern that is linked with HRT. In fact, it is claimed that combined HRT with estrogen plus progestin is a cause for breast cancer. However, while followup analysis approximately three years after termination of the WHI study demonstrated an increased risk for "all-cause cancer" for participants in the CEE + MPA trial compared to the placebo group [158], the risk for breast cancer and other types of cancer did not differ between groups. Similarly, recent retrospective analyses of the WHI data found insufficient evidence that estrogen plus progestin increased risk of breast cancer [159]. Another study using the WHI data found that among women in the CEE + MPA trial, increased breast cancer risk was especially pronounced among women with breast tenderness [160]. In fact, new onset of breast tenderness after HRT initiation was associated with increased breast cancer risk among women assigned to the CEE + MPA trial, but not among women assigned to CEE-alone. In contrast, an additional followup analyses after the termination of the WHI data demonstrated that participants in the CEE-alone trial did not demonstrate increased risk for breast cancer [161]. Although the available information is insufficient at this time to support a clear link between HRT and increased risk for breast cancer, at least one study from the WHI has reported an increased risk of breast cancer among users of estrogen plus progestin with new onset of breast tenderness. This is an issue that requires continued investigation.

In clinical settings, the financial cost will need to be considered when recommending E2-based therapies for prevention of AD or other neurodegenerative diseases. Patients and their physicians will have to determine whether the potential cognitive benefit associated with E2 will outweigh the financial cost, as well as the above-mentioned medical risks.

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*Review Article*

# The Complexity of Sporadic Alzheimer's Disease Pathogenesis: The Role of RAGE as Therapeutic Target to Promote Neuroprotection by Inhibiting Neurovascular Dysfunction

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Alzheimer's disease (AD) is the most common cause of dementia. Amyloid plaques and neurofibrillary tangles are prominent pathological features of AD. Aging and age-dependent oxidative stress are the major nongenetic risk factors for AD. The beta-amyloid peptide (A$\beta$), the major component of plaques, and advanced glycation end products (AGEs) are key activators of plaque-associated cellular dysfunction. A$\beta$ and AGEs bind to the receptor for AGEs (RAGE), which transmits the signal from RAGE via redox-sensitive pathways to nuclear factor kappa-B (NF-$\kappa$B). RAGE-mediated signaling is an important contributor to neurodegeneration in AD. We will summarize the current knowledge and ongoing studies on RAGE function in AD. We will also present evidence for a novel pathway induced by RAGE in AD, which leads to the expression of thioredoxin interacting protein (TXNIP), providing further evidence that pharmacological inhibition of RAGE will promote neuroprotection by blocking neurovascular dysfunction in AD.

## 1. Introduction

Alzheimer's disease (AD) pathology is characterized in by the presence of several kinds of amyloid plaques and neurofibrillary tangles in the brain, which are mainly composed by the beta amyloid (A$\beta$), derived from the proteolytic cleavage of the amyloid precursor protein (APP), and hyperphosphorylated tau [1]. AD can be subdivided in 2 major forms: (i) familial AD, which represents rare early onset forms due to gene mutations leading to enhanced A$\beta$ production or faster aggregating A$\beta$ peptide; (ii) sporadic AD forms, which represent about 95% of AD cases [2]. The pathogenesis of sporadic AD is extremely complex, and its ultimate cause is still under debate. Epidemiological studies reveal growing evidence that most cases of sporadic AD likely involve a combination of genetic and environmental risk factors. However, the only risk factors so far validated for late-onset disease are age, family history, and the susceptibility gene ApoE4 allele [3].

A hallmark of the aged brain is the presence of oxidative stress [4]. A$\beta$ fibrils are toxic by generating oxygen free radicals in the absence of any cellular element [5, 6]. However, synaptic dysfunction and behavioral changes in AD precede the formation of large A$\beta$ aggregates and fibrils. Indeed, A$\beta$ dimers and soluble oligomers are considered the major toxic form [7, 8], while fibrils-induced oxidative stress operates late in the course of AD. Thus, the mechanisms through which A$\beta$ exerts its toxic effect at the early stages of AD remain still to be clarified. Recent evidences suggest that age-relate cofactors play a key function in mediating the toxicity of A$\beta$ at early, AD stages. One of the risk factors is diabetes mellitus (DM) and several studies demonstrated a link between DM and AD [9–11]. In agreement, both hyperglycemia in DM and age-dependent oxidative stress induce the formation of advanced glycation end products (AGEs) [12, 13]. AGEs derive from a multistep reaction of reducing sugars or dicarbonyl compounds with the amino groups of proteins [13]. AGEs accumulate in AD brain and

accelerate A$\beta$ deposition [14, 15]. It has been shown that the interaction of AGEs with their receptor (RAGE) induces the production of reactive oxygen species (ROS), participating to the early toxic events that lead to AD progression [16]. RAGE is a multiligand receptor of the immunoglobulin superfamily of cell surface molecules acting as counterreceptor for various ligands, such as AGEs, S100/calgranulins, HMGB1 proteins, A$\beta$ peptides, and the family of beta-sheet fibrils [17, 18]. Its ectodomain is constituted by one V-type followed by two C-type domains. The N-terminal V-domain seems to be implicated in the recognition of RAGE ligands [19]. Studies with RAGE−/− mice confirmed that RAGE contributes to AD [20, 21]. Notably, diabetic AD patients show enhanced cell damage, which is RAGE dependent [11]. Thus, RAGE seems to represent an excellent cofactor promoting A$\beta$-induced cellular dysfunction.

Several studies indicate that RAGE induces neurodegeneration in AD via multiple pathways. In AD brain, RAGE is evident in neurons, microglia, astrocytes, and in brain endothelial cells [19, 22]. The activation of RAGE expressed in neuronal cells promotes synaptic dysfunction. RAGE also promotes neurodegeneration by inducing inflammation in glial cells. Moreover, RAGE is responsible of the transport of A$\beta$ from the blood to the brain [23], inducing cerebrovascular dysfunction that ultimately results in neurovascular inflammation and subsequent synaptotoxicity [24]. Notably, the G82S RAGE allele (a polymorphism in RAGE sequence) is associated with increased risk of AD [25], supporting the hypothesis that RAGE is implicated in the progression of sporadic AD. At early stages of AD, when the level of A$\beta$ and AGEs are low, RAGE amplifies their effects on different cell types, ultimately contributing to neuronal dysfunction and neurodegeneration. Different animal models have been analyzed to decipher the role of RAGE in AD progression: (i) injection of AGEs into the rat hippocampus; (ii) injection of A$\beta$ in rat hippocampus; (iii) various transgenic (Tg) mice expressing one or more gene variant of the amyloid precursor protein (APP); (iv) presenilins, which are implicated in APP cleavage and A$\beta$ production leading to amyloid plaque formation; (v) tau that forms the characteristic tangles when is hyperphosphorylated. In addition, the brain of animal model of diabetes was analyzed to find the link between DM and AD.

We recently demonstrated that RAGE triggering induces the expression of thioredoxin interacting protein (TXNIP) in various cell types, promoting inflammation [26, 27]. TXNIP binds to thioredoxin (TRX) and inhibits its antioxidant activity, leading to oxidative stress in various cell type [28]. We demonstrated that oxidative stress plays a key function in AD progression [6, 29]. TXNIP expression is enhanced in several disease risk for AD: diabetes [26, 28, 30], hypertension [31], and ischemia [32]. Insulin is necessary to maintain normal brain function, and peripheral insulin resistance enhances the risk to develop AD, by affecting brain glucose metabolism, neurotransmitters levels, enhancing inflammation [33]. Interestingly, TXNIP is necessary to mediate insulin resistance in diabetes [34]. TXNIP is early overexpressed in the hippocampus of an AD mice model. Moreover, A$\beta$ induces the RAGE-dependent expression of TXNIP in an in vitro model of the blood brain barrier (BBB).

Notably, TXNIP and RAGE, both may exacerbate injury and inflammation when chronically activated, while they mediate neuronal repair when transiently expressed [26, 27]. Moreover, RAGE can also promote neurite outgrowth [35]. Thus, inhibition of chronic activation of RAGE and TXNIP can efficiently provide neuroprotection in AD.

## 2. Role of RAGE in Amplifying Age-Dependent Oxidative Stress

Human aging is an inexorable biological phenomenon characterized by a progressive decrease in physiological capacity, and the reduced ability to respond to environmental stresses leads to increased susceptibility to disease. In 1956, Harman developed the free radical theory of aging [36] that argues that aging results from the damage generated by reactive oxygen species (ROS) [37]. According to this theory, aging is the result of accumulation of oxidative-damaged macromolecules (lipid, protein, DNA) due to the aerobic metabolism, which accumulate throughout lifetime [38]. Thus, aging is associated with imbalance between the rate of antioxidant defenses and intracellular concentration of ROS. The relevance role of ROS in aging consists in their ability to attack vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids. These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, and inhibition of protein synthesis, DNA damage, ultimately resulting in cell death. Many disorders, like cardiovascular diseases, rheumatoid arthritis, cancer, atherosclerosis, and AIDS, have been reported as the ROS-mediated disorders.

ROS has been also implicated in neurodegenerative diseases like Parkinson and Alzheimer diseases (AD). Indeed, the brain is particularly vulnerable to oxidative damage because of its high utilization of oxygen, increased levels of polyunsaturated fatty acid, and relatively high levels of redox transition metal ions; in addition, the brain has relatively low levels of antioxidants [39]. The presence of iron ion in an oxygen-rich environment can further lead to enhanced production of hydroxyl free radicals and ultimately lead to a cascade of oxidative events [6]. In the AD brain, the role of ROS has been well documented with markers for protein, DNA, RNA oxidation, and lipid peroxidation. In fact, increased reactive carbonyls were the first form of oxidative damage identified in AD [40]. Several studies showed the presence of additional protein markers like protein nitration supporting that nitrosative stress also contributes to neurodegeneration disease [39]. Amplified lipid peroxidation has been also described in several neurodegenerative diseases [41]. AD brains show an increase in free 4-hydroxy-2-trans-nonenal (HNE) in amygdala, parahippocampal gyrus, and hippocampus of the AD brain compared with age-matched controls [42]. In addition, DNA is a target of ROS, which leads to cellular aging. Oxidative damage to DNA induces strand breaks DNA-DNA and DNA-protein cross-linking and translocation. DNA bases are also attacked by the lipid peroxidation. This modification can

cause inappropriate base leading to alter protein synthesis [43]. AGEs are considered important markers of oxidative stress and accumulating during aging and diseases, markers of carbonyl stress, which accumulate due to an increased level of sugars and reactive dicarbonyl compounds such as glucose, fructose, deoxyglucose, glyoxal, methylglyoxal, and triosephosphates [38, 44]. AGE formation begins when amino groups of proteins particularly the N-terminal amino group and side chains of lysine and arginine react nonenzymatically with these reactive carbonyl compounds [45]. This posttranslational modification, termed "non-enzymatic glycation" or "glycation," derives from reversible Schiff-base adducts to protein through oxidations and dehydrations bound Amadori products. The irreversible formation of AGEs results in protease-resistant cross-linking of peptides, proteins, and other macromolecules. AGEs are localized in pyramidal neurons that appear to selectively accumulate AGEs in an age-dependant manner. In the AD brain, AGE colocalize with activated astrocytes [46]. In 2011, Srikanth et al. showed that the percentage of AGE positive neurons and astroglia increase in Alzheimer with the progression of disease, which might contribute to many aspects of neuronal dysfunction in AD by processes, such as inflammatory activation of microglia, or direct cytotoxicity via formation of free radicals [45], presumably mediated through activation of their receptor RAGE [45]. RAGE binds also the monomeric and fibrillary forms of A$\beta$. Upon binding of ligands (AGEs and A$\beta$), RAGE triggers intracellular signaling pathways via phosphatidylinositol-3 kinase, Ki-Ras, and mitogen-activated protein kinases, the Erk1 and Erk2 [17]. Those pathways culminate in the activation of the transcription factor nuclear factor kappa B (NF-$\kappa$B) and subsequent transcription of a number of genes, including endothelin-1, tissue factor, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-$\alpha$ [17, 18, 47]. Activation of NF-$\kappa$B and induction of cytokines can also contribute to neuronal plasticity and the cellular response to neurodegeneration [48]. RAGE-induced signaling results in an initial neuroprotective effect [27], while it contributes to cellular dysfunction when chronically activated [17]. Notably, NF-$\kappa$B induces the expression of RAGE, leading to a positive loop, which amplify the cellular response to external stress [17]. Furthermore, the engagement of RAGE by AGEs triggers the generation of ROS via the activation of NADPH oxidase (NOX) [45]. NOX catalyzes the reduction of molecular $O_2$ by donating an electron from reduced nicotinamide adenine dinucleotide phosphate to generate superoxide. NOX plays an important role in AD-induced ROS release. Thus, RAGE can be considered a key mediator of age-induced oxidative stress by its capability to amplify a stress signal, contributing to the progression of neurodegenerative processes in sporadic AD.

## 3. Role of Neuronal RAGE in AD

The expression level of RAGE is high in rodent cortical neurons during the neonatal period [49], while its presence strongly decreases during maturity with few cortical neurons showing RAGE staining [50]. However, increased RAGE expression in the brain parallels the progression of neurodegenerative diseases such as AD and Huntington's disease [11, 21, 50, 51]. Notably, AD patients show enhanced RAGE, A$\beta$, and AGEs expression in the whole hippocampus, especially in dentate gyrus neurons and in CA3 pyramidal neurons, which parallels the impairment of short-term memory that is characteristic of AD due to neuronal dysfunction in the hippocampus [11].

Chronic activation of RAGE affects neuronal function by activating various signaling pathways, promoting both the phosphorylation of tau and the production of A$\beta$, as well as it mediates A$\beta$ toxicity.

A recent study demonstrates that injection of AGEs in the rat hippocampus leads to RAGE-dependent tau hyperphosphorylation, spatial memory deficit, and impaired synaptic transmission as demonstrated by inhibition of long-term potentiation (LTP) in AGEs treated rats [52]. Altered synaptic transmission correlated with RAGE-dependent tau hyperphosphorylation that is due to inhibition of Akt and subsequent activation of GSK3. RAGE activation leads also to alterations of the postsynaptic machinery and decreased density of dendritic spines [52]. Interestingly, AD is also characterized by nonenzymatically glycated tau [53], which induces neuronal oxidative and subsequent release of A$\beta$, further supporting the role of metabolic dysfunction in sporadic AD.

RAGE induces the expression of BACE 1, a key enzyme implicated in the production of A$\beta$ after stimulation with either AGEs or A$\beta$. RAGE triggering leads to NF-$\kappa$B nuclear translocation, which in turn enhances the expression of RAGE leading to a vicious circle producing RAGE-dependent cellular dysfunction [17, 18, 47]. In the brain of a rat model of diabetes, activation of RAGE with AGEs leads to NF-$\kappa$B-dependent expression of BACE1 [16]. AGEs are increased in the brain of AD patients [16]. These results confirm the role of AGEs and RAGE as molecules linking DM and AD. Another study demonstrated that RAGE induces BACE1 expression in an AD mice model and in A$\beta$-stimulated neuronal cells in vitro, by stimulating intracellular calcium and activating nuclear factor of activated T cell 1 (NFAT) [54]. Although the signaling pathway induced by RAGE upon A$\beta$ stimulation differs compared to the study describing the role of AGEs-stimulated RAGE in the DM animal model, both reports underline the role of RAGE in promoting the expression of BACE1, which enhances A$\beta$ production in the brain.

Several evidences clearly demonstrate that RAGE strongly enhances A$\beta$-induced neuronal dysfunction in AD transgenic (Tg) mice that overexpress a mutant form of human amyloid precursor protein (mAPP), which enhances the production of A$\beta$1-42 in neuronal cells. These mice show A$\beta$-induced synaptotoxicity in the absence of amyloid plaque [55]. Overexpression of RAGE anticipates the onset of neuronal dysfunction in double transgenic mice overexpressing neuronal mAPP and RAGE (Tg mAPP/RAGE) compared to the single Tg expressing mAPP only [56]. RAGE-dependent anticipation of neuronal dysfunction was demonstrated by earlier impairment of learning/memory in double Tgs mAPP/RAGE compared to

single Tg mAPP mice. Exacerbation of memory impairment correlates with an anticipation of synaptic dysfunction in the hippocampus of double Tgs as demonstrated by alteration of LTP [56]. A decrement of cholinergic fibers and presynaptic terminals appears earlier in mAPP/RAGE compared to map mice [56]. On the contrary, inhibition of RAGE confers a neuroprotective effect in AD mice, as demonstrated in double Tg mice expressing mAPP and a dominant negative form of RAGE (DNRAGE) in neurons [56]. DNRAGE encodes for a truncated form of RAGE lacking the intracellular domain necessary to induce RAGE-mediated signaling, while maintaining the extracellular domain for ligand binding. DNRAGE expression blocks the function of endogenous RAGE [56]. Double Tg mAPP/DNRAGE performed better in learning and memory test compared to single Tg mAPP. Expression of DNRAGE completely prevented neuropathologic changes such as loss of cholinergic fibers induced by mAPP [56].

Another area of the brain that is important in memory process and is early affected in AD is the entorhinal cortex. In agreement, oligomeric A$\beta$1-42 impairs LTP in slides derived from this brain area of wild-type (wt) mice [57]. A$\beta$-induced LTP alteration is inhibited by coaddition of anti-RAGE IgG. Similarly, A$\beta$ has not any effect on slides derived from RAGE null mice or Tg mice expressing neuronal DNRAGE [57]. Moreover, this study demonstrated that RAGE is implicated in A$\beta$-induced synaptic dysfunction by activating the pathway of p38 MAPK [57, 58]. RAGE plays a key role also in A$\beta$-dependent inhibition of synaptic plasticity in intracortical circuits of the visual cortex, and RAGE blockade confers a neuroprotective effect against A$\beta$-induced neuronal dysfunction [59]. In contrast, in Arc/swe AD mice, which overexpress hAPP carrying the Swedish (swe) mutation, which enhances A$\beta$ production, and the arctic (arc) mutation in A$\beta$ sequence, which leads to a faster aggregation of A$\beta$ [60], the knockout of RAGE has only a minimal effect on A$\beta$ load and does not ameliorate synaptic dysfunction. Taken together, these data underline the differences in the pathologic mechanisms implicated in sporadic and familial AD, supporting the hypothesis that RAGE plays a key function specifically in the progression of sporadic AD.

Several studies demonstrated that A$\beta$ and AGEs affect energy metabolism by decreasing mitochondrial activity and induce neurodegeneration by producing mitochondrial damage [19, 61]. Injection of A$\beta$25-35 toxic fragment in rat CA1 hippocampus enhances RAGE expression in CA1, which parallels with a 56% decrement in mitochondrial activity and the presence of neurodegenerative events [62]. RAGE is involved in the uptake of A$\beta$ and A$\beta$ targeting to mitochondria in cortical neurons, leading to a decrement in the activity of a key mitochondrial respiratory enzyme, the cytochrome c oxidase (COX IV) [63]. Blockade of RAGE with anti-RAGE IgG or A$\beta$ treatment of neurons derived from RAGE null mice diminishes A$\beta$ targeting to mitochondria and subsequent mitochondrial dysfunction. Inhibition of RAGE-dependent p38 MAK activation blocks A$\beta$ targeting to mitochondria and the subsequent mitochondrial damage. RAGE colocalizes with A$\beta$ in an intracellular compartment *in vivo* in pyramidal cells of the CA3 region of the hippocampus in the Tg mAPP mice [63] further supporting the role of RAGE in A$\beta$-mediated neurodegeneration by affecting mitochondrial function. Moreover, these studies demonstrate that RAGE inhibition confers a neuroprotective effect against A$\beta$-mediated toxicity.

Several studies demonstrated that RAGE triggering induces neurite outgrowth and neuronal differentiation [35, 64–69]. Furthermore, various studies including our own demonstrate that RAGE is required for the repair of the injured nerve [27, 70, 71]. Thus, RAGE plays a dual function: it can mediate neurite outgrowth and neuronal repair, while it induces neuronal dysfunction when chronically activated. Because of the dual function of RAGE, compounds capable to block the chronic activation of RAGE can exert a neuroprotective effect in AD.

## 4. Role of RAGE in Glial Cells and Inflammation in AD

Several evidences substantiate the association between neuroinflammatory mechanisms and the pathological events leading to neuronal dysfunction and neurodegeneration. The brain of AD patients shows chronic inflammation that is characterized by the presence of reactive astrocytes and activated microglia [72]. In healthy physiological conditions, astrocytes are necessary to maintain brain homeostasis and neuronal function. They provide metabolic support for neurons in form of lactate, glutamate uptake and its conversion into glutamine, and synthesis of antioxidant enzymes [72]. Microglial cells represent the innate immune system in the brain as they can have a role as cerebral macrophages as well they recruit and stimulate astrocytes [73]. Neuroinflammation and microglial activations regulate the delicate balance of immune response and neuronal homeostasis. The innate immune responses of glial to injurious insults or activating stimuli lead to beneficial outcomes, such as phagocytosis of pathogens, and production of reparative and protective factors. However, chronic activation of glial cells results in overproduction of proinflammatory factors, disturb homeostasis, and ultimately exacerbates neuronal dysfunction enhancing the progression of neuropathology [74]. Activated astrocytes in AD fails in providing metabolic support to neurons, contributing in inducing neurodegeneration [72]. Moreover, the activation of astrocytes and microglia leads to chronic oxidative stress in AD patients, further contributing to neurodegenerative processes [72]. Noteworthy, oxidative stress leads to the formation of AGEs, which will activate RAGE [72]. Several studies including our own demonstrated that activation of RAGE induces oxidative stress and inflammation [18, 26, 27, 47, 75, 76]. Thus, glial inflammation and subsequent AGEs formation in the presence of A$\beta$ lead to a positive feedback loops by which inflammation in AD increases proinflammatory signaling. Inflammation enhances the processing of APP in astrocytes by inducing BACE1 expression, leading to A$\beta$ deposition, further activating RAGE [45]. Moreover, RAGE ligands enhance the expression of RAGE itself, leading of a positive loop that induces the expression of RAGE and

subsequent oxidative stress and inflammation, which in turn sustain the formation of AGEs and A$\beta$ [17]. Interaction of A$\beta$ with RAGE results in increased expression of macrophage colony stimulating factor (M-CSF) in neuronal cells [77]. Stimulation of microglia by M-CSF results in enhanced cell survival in cell stress conditions, proliferation and induction of proinflammatory gene expression, which leads to chronic inflammation and contributes to neurodegenerative processes [77]. Indeed, M-CSF induces cell survival in microglial cells, which express c-fms receptor. On the contrary, neuronal cells do not express c-fms receptor and do not benefit of M-CSF prosurvival effects, while they are further affected by the proinflammatory reaction of glial cells [19]. The combination of AGEs and A$\beta$ synergistically induces the expression of proinflammatory cytokines, such as TNF-$\alpha$, IL-6, and M-CSF [45]. Moreover, A$\beta$ induces the expression and secretion of IL-1$\beta$ in glial cells [45] via RAGE [27]. RAGE is overexpressed in the microglial cell in AD patients [78] and in an AD mice model (mAPP Tg) [56]. Activated microglia exacerbate A$\beta$-induced neuronal toxicity [74], and RAGE is a key mediator of activated microglial effects in AD neuronal dysfunction [78, 79]. Targeted overexpression of RAGE in the microglia of mAPP Tg mice (double Tg mAPP/micRAGE) enhances the expression of proinflammatory cytokines, increases A$\beta$ production, and accelerates neuropathologic changes compared to single Tg mAPP, as demonstrated by anticipation of cholinergic fiber loss and alteration in learning and memory [78]. Conversely, targeted overexpression of a dominant negative form of RAGE in microglia of mAPP Tg mice (double Tg mAPP/micDNRAGE) leads to a decrement of cytokines and A$\beta$ production and ameliorates neuronal dysfunction compared to the single Tg mAPP [78]. In addition, targeted overexpression of a dominant negative form of RAGE in microglia (double Tg mAPP/micDNRAGE) attenuates A$\beta$-induced synaptic dysfunction and A$\beta$-dependent inhibition of long-term depression (LTD) in entorhinal cortex [79], demonstrating that RAGE blockade inhibits A$\beta$-induced neuronal dysfunction.

In summary, several studies support the hypothesis that RAGE-mediated inflammation in AD contributes in inducing neuronal dysfunction. On the contrary, these studies demonstrate that inhibition of RAGE activation induces neuroprotection and ameliorates AD progression.

## 5. Role of RAGE and Vascular Dysfunction in AD

The potential link between cerebral blood vessel disease and Alzheimer's is one promising area of research. Vascular disease in the aged appears to have strong implications for neurodegeneration leading to dementia. Preliminary studies indicate that a broad spectrum of cerebrovascular lesions could lead to a decline in cognitive function. Moreover, nearly 80 percent of individuals with AD also have cardiovascular disease at autopsy, supporting the hypothesis that systemic vascular factors are risk factors for developing AD. This risk encompasses different forms of cardiovascular disease, including coronary artery disease, carotid atherosclerosis, history of hypertension or high cholesterol, type II diabetes, and stroke or transient ischemic attacks [3]. Indeed, another hypothesis accounting for the pathogenesis of AD is the impairment of the blood brain barrier (BBB) [23]. Cerebral blood vessels undergo profound changes with aging and in AD [80]. The BBB blocks the free diffusion of circulating molecules, leukocytes, and monocytes into the brain interstitial space. Moreover, the BBB plays a key role in regulating the glial and neuronal environment. The BBB is constituted by endothelial cells fused by high-resistance tight junctions, in order to separate the blood from the brain. The disruption of the tight junctions affects the regulated transport of molecules and monocytes between blood and brain and brain and blood and induces angiogenesis and vessels regression, as well as brain hypoperfusion and inflammation, promoting ultimately synaptic dysfunction and neurodegeneration. Alterations of the BBB, vascular density, fragmentation of vessels, alteration of the basement membranes, and a decrement of mitochondria in the BBB occur in AD [80]. Notably, BBB dysfunction is associated to several risk factors for AD, such as stroke, cerebrovascular ischemia, hypertension, and mutation in the ApoE gene, which represents the only validated genetic risk factor of AD [3]. Since the large majority of AD cases are sporadic, it has been recently hypothesized that the accumulation of A$\beta$ into the brain and around blood vessels is due in an alteration of clearance of A$\beta$ from the brain and an enhanced transport of A$\beta$ into the brain [22]. In agreement, Tg2576 AD mice display enhanced BBB permeability compared to control mice at 4 months of age, before the appearance of plaque deposition and memory impairment [81]. A correlation between BBB dysfunction and AD has been demonstrated in AD patients. Noteworthy, BBB impairment in these patients was not associated with vascular diseases risk for AD, suggesting that the mechanisms inducing BBB alterations in AD differ from that one implicated in vascular dementia [82].

RAGE is upregulated in AD brain vasculature [10, 11, 50]. *In vivo* studies show a RAGE-dependent transport of A$\beta$1-40 and A$\beta$1-42 into the hippocampus and cortex, which is inhibited by anti-RAGE blocking antibodies. The transport of A$\beta$ is strongly impaired and undetectable in RAGE null mice [23]. RAGE-mediated transport of A$\beta$ leads to neurovascular stress, induction of the expression of TNF-$\alpha$ and IL-6, which are detected mostly at the level of neurons. Notably, infusion with physiological concentration of A$\beta$ (50 pM) does not induce the expression of proinflammatory cytokines, while neurovascular inflammation is detected when pathological concentrations of A$\beta$ (4.5 nM) are infused in the mice [23]. Moreover, A$\beta$-RAGE interaction on the BBB induces vasoconstriction by promoting the expression of endothelin-1. Notably, infusion of anti-RAGE IgG ameliorates vascular dysfunction and blocks endothelin-1 expression in Tg2576 AD mice [23].

It has been demonstrated that blood or BM-derived monocytes infiltrate the AD brain, enhancing inflammation [83]. Antibodies against RAGE inhibit A$\beta$-induced monocytes transmigration across the BBB [84], further demonstrating the key role of RAGE in promoting neurovascular inflammation in AD. Thus, RAGE expressed in brain

microvessels participates in AD by enhancing A$\beta$-transport across the BBB and promoting neurovascular inflammation. Conversely, inhibition of RAGE is beneficial by blocking A$\beta$ transport across the BBB and the subsequent inflammatory response.

## 6. RAGE-TXNIP Axis: Evidence of a Novel Pathway Induced by RAGE in AD

Recent studies using the human brain indicate that insulin signaling is impaired in the AD brain. In neurons, this insulin signaling plays a key role in modulating synaptic function and neuronal senescence [85]. Spatial learning in rats induces the expression of insulin receptor and of insulin receptor substrate 1 (IRS 1) in the hippocampus. Moreover, insulin regulates tau phosphorylation, a hallmark of AD [86]. Insulin also regulates glucose metabolism in the brain by modulating the expression of glucose transporters [85]. TXNIP is an intriguing candidate molecule that may provide a common link between brain insulin resistance and AD. TXNIP was initially characterized for its capability to inhibit thioredoxin, leading to oxidative stress [26, 87]. However, recent studies demonstrated that TXNIP regulates glucose metabolism [88, 89], and its expression is associated to the senescence process [90]. Notably, TXNIP null mice are resistant to diabetes, showing that TXNIP is necessary for the induction of insulin resistance [34]. In the mice brain, TXNIP is expressed in the nuclei of astrocytes and at low level in some neurons. TXNIP expression is low in the hippocampus, while it is expressed constitutively in hypothalamic neurons where it senses nutrients excess [91, 92]. TXNIP is also an early induced gene by apoptosis in cerebellar neurons [93]. Insulin modulates memory by promoting the expression of N-methyl-D-aspartate (NMDA) receptors, which enhances neuronal $Ca^{2+}$ influx, consolidating neuronal synaptic association and promoting LTP [85]. Synaptic activity inhibits TXNIP expression in neurons through NMDA receptor (NMDAR) activation. Blockade of NMDAR enhances TXNIP expression, promoting neuronal vulnerability to oxidative damage [94]. Notably, A$\beta$ affects NMDAR function and trafficking [95], further supporting the hypothesis that TXNIP may be implicated in AD. However, no any study up to now investigated TXNIP expression in AD. For this reason, we analyzed the expression of TXNIP in the brain of the 5xFAD mice model of AD. 5xFAD expresses neuronal human APP carrying three AD familiar mutation (Swedish, Florida, London) and presenilin 1 (PS1) containing 2 mutations (M146L and L286V) [96]. Since TXNIP is implicated in senescence, we used the 5xFAD mice that display an early AD phenotype. Indeed, 5xFAD mice show intraneuronal A$\beta$ accumulation at 2 months age, impaired learning/memory and reduction of synaptophysin levels at 4 months age, and cortical neuronal apoptosis at 9 months age [96]. TXNIP was overexpressed in the hippocampus (Figure 1 top and middle) and in the entherinal cortex (*not shown*) of 5xFAD mice at 6 months of age compared to control mice. To investigate TXNIP expression, we used a mouse anti-TXNIP monoclonal antibody (clone JY2, MBL). Similar results were obtained using a rabbit anti-TXNIP polyclonal antibody (Invitrogen). TXNIP overexpression paralleled enhanced astrogliosis, as demonstrated by increased expression of glial fibrillary acidic protein in the hippocampus (Figure 1 bottom). The expression of TXNIP in 5xFAD brain capillary endothelial cells in the hippocampus was detected using both monoclonal and the polyclonal anti-TXNIP antibodies (*not shown*). Noteworthy, hippocampus and entorhinal cortex are associated to the early learning/memory impairment in AD. Since we previously demonstrated that RAGE induces TXNIP expression in retinal endothelial cells leading to chronic inflammation and ultimately inducing neurodegeneration in diabetic retina [26, 30], we studied whether A$\beta$ induces TXNIP expression in brain derived endothelial cells (RBE4). RBE4 cells were maintained in differentiation medium (F10/MEM, 2.5% FCS, hydrocortisone 14 $\mu$M, Hepes 10 mM, bFGF 1 $\mu$g/mL) [97] for 5 days, before stimulated for 6 h with A$\beta$b1-42 (3 $\mu$M). Since hyperglycemia (HG) induces TXNIP expression [26, 87], as control we stimulated RBE4 cells for 6 h with HG (25 mM glucose). Both HG and A$\beta$ induced TXNIP expression in RBE4 cells (Figure 2(a)). A$\beta$-induced TXNIP expression was RAGE-dependent, because an anti-RAGE blocking antibody (R&D system) [98] completely inhibited A$\beta$-induced TXNIP expression in RBE4 cells (Figure 2(b)). Moreover, RBE4 cells treated for 6 h with either HG (25 mM) or A$\beta$ (3 $\mu$M) displayed enhanced RAGE expression compared to control cells (Figure 2(c)). It has been recently shown that TXNIP translocation to the plasma membrane in endothelial cells participates in cell migration leading to angiogenesis [99]. Since angiogenesis occurs in AD [80], we investigated whether A$\beta$ treatment induces TXNIP translocation in RBE4 cells. Fractionation analysis of cell extracts reveals that 45 min of A$\beta$ treatment increases the cofractionation of TXNIP with the plasma membrane marker VE-cadherin (Figure 3(a)). This result was confirmed by immunofluorescence analysis of TXNIP subcellular localization in the absence or presence of A$\beta$ treatment, which displays an enhanced colocalization of TXNIP with VE-cadherin following A$\beta$ treatment (Figure 3(b)). We also observed an enhanced cofractionation of TXNIP with the cytoskeletal fraction following A$\beta$ treatment (Figure 3(a)), which is confirmed by immunofluorescence analysis showing enhanced colocalization of TXNIP with actin following A$\beta$ treatment (data not shown). Notably, it has been recently demonstrated that triggering of RAGE in endothelial cells leads to altered actin reorganization and membrane resealing, participating in vascular dysfunction [100].

These data strongly imply that RAGE-TXNIP axis contributes to vascular dysfunction in AD, suggesting that RAGE-TXNIP axis is a novel therapeutic target to ameliorate AD.

## 7. Pharmacological Treatment to Ameliorate AD Progression by Blocking RAGE

Since RAGE is implicated in AD progression by orchestrating cellular dysfunction in various cell types, a pharmacological treatment aimed to inhibit RAGE chronic activation would

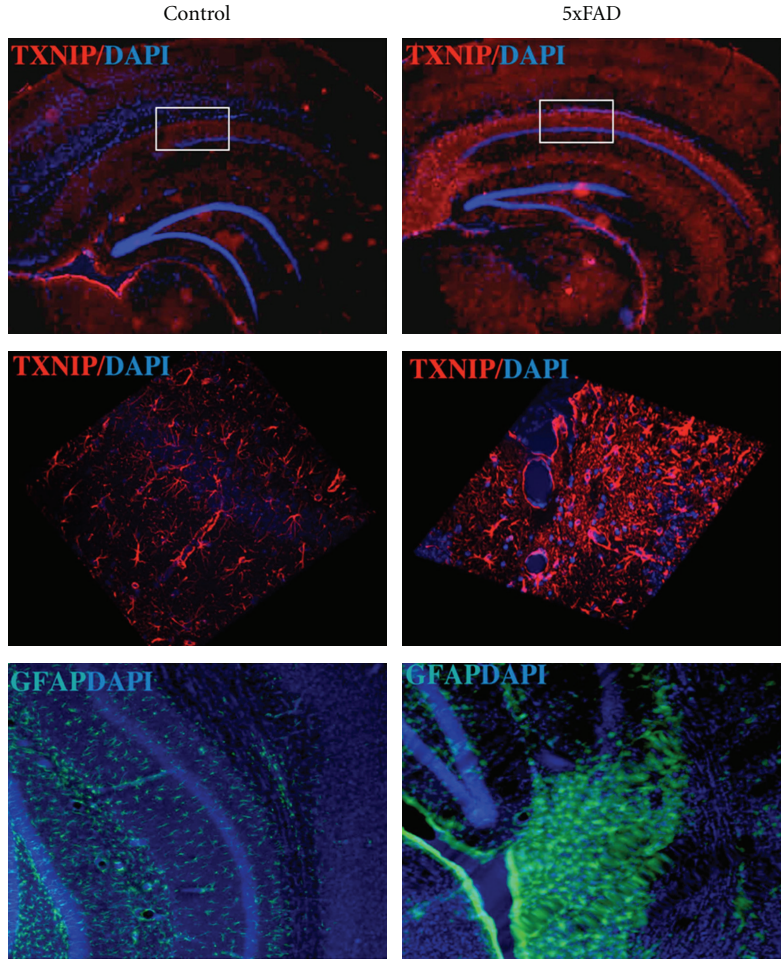


Figure 1: TXNIP is overexpressed in the hippocampus of the 5xFAD mice. Top: Floating brain slices were incubated 24 h with mouse anti-TXNIP monoclonal antibody in PBS 3% BSA, 0.1% Triton X-100 (blocking) at 4°C. Slides were washed 3 times for 15 min with PBS and incubated for 45 min with TRITC-conjugated secondary antibody (red). Nuclei were stained by incubating the slides with Hoecst (blue) together with the secondary antibody. Slides were mounted using mounting medium and analyzed with confocal microscopy (Zeiss). Center: Confocal analysis and 3 dimensional reconstruction (Zen software of Zeiss) of TXNIP staining in the hippocampus. Bottom: Floating brain slices were incubated 2 h at room temperature with rabbit anti-GFAP polyclonal antibody in PBS 3% BSA, 0.1% Triton X-100 (blocking). Slides were washed 3 times for 15 min with PBS and incubated for 45 min with FITC-conjugated secondary antibody (green). Slides were mounted using mounting medium and analyzed with confocal microscopy (Zeiss). These results are representative of 4 independent experiments (4 animals).

be beneficial in ameliorating AD. The small molecule PF-04494700 inhibits RAGE by blocking the interaction of the receptor with its ligands such as A$\beta$, AGEs, HMGB1, and members of the proinflammatory S100 family members [101]. Thus, PF-04494700 was thought to be capable to ameliorate AD by inhibiting both inflammation and A$\beta$-induced neurodegeneration. An initial 10-week-long phase 2 safety trial demonstrated a good safety profile of PF-04494700 in AD patients, even if there was not significant clinical amelioration during the short observation period [101]. Thus, a long-term clinical trail was initiated with three group of treatment: one group received placebo, the second 20 mg/day of PF-04494700, and the third 5 mg/day of the drug, and the researcher analyzed Alzheimer's Disease Assessment-cognitive subscale (ADAS-cog) score, safety indicators, additional cognitive tests, structural magnetic resonance imaging (MRI) measurements, A$\beta$ imaging by positron emission tomography (PET), and levels of the biomarkers A$\beta$ and tau in cerebrospinal fluid (CSF). However, the trial was discontinued after 12 months because the highest dose of PF-04494700 resulted in worsening the ADAS-cog score and side effects, while the lower dose was safe (see Alzheimer Research Forum article: "Door Slams on RAGE" 9th November 2011 http://www.alzforum.org/new/detail.asp?id=2960). Therefore, the use of this drug to ameliorate AD is still debatable. Although the clinical trial was abandoned, the researchers continued to follow the patients and they collected data obtained from visiting these patients after 18 months from the start of the trial. When Douglas Galasko presented the completed data set during the 4th International Conference on Clinical Trials on Alzheimer's Disease (CTAD; November 3–5, 2011, in San Diego, CA, USA), he notably

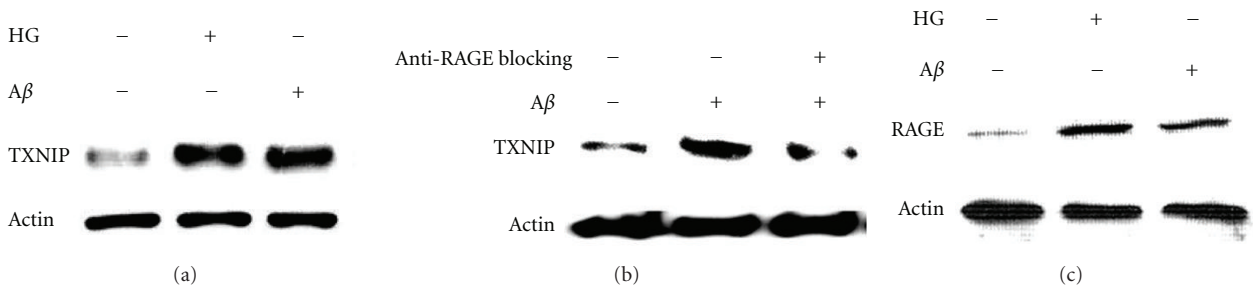


Figure 2: Aβ induces RAGE-dependent TXNIP expression in RBE4 brain endothelial cells. RBE4 cells were maintained 5 days in differentiation medium (F10/MEM, 2.5% FCS, hydrocortisone 14 μM, Hepes 10 mM, bFGF 1 μg/mL). RBE4 cells were stimulated for 6 h with either Aβ1-42 (3 μM) or HG (25 mM) in differentiation medium. Cells were lysed in RIPA buffer. TXNIP expression was analyzed by western blotting using a mouse anti-TXNIP monoclonal antibody (MBL). Protein loading was analyzed by western blotting of actin. (b) RBE4 cells were maintained as described in (a) and stimulated for 6 h with either Aβ1-42 (3 μM) both in the absence or presence of an anti-RAGE blocking antibody (R&D system). TXNIP expression and protein loading were analyzed by western blotting as in (a). (c) RBE4 cells were maintained as described in (a) and stimulated for 6 h with either Aβ1-42 (3 μM) or HG (25 mM) in differentiation medium. RAGE expression was analyzed by western blotting using a rabbit anti-RAGE polyclonal antibody (Santa Cruz). Protein loading was analyzed by western blotting of actin. These data are representative of 3 independent experiments.

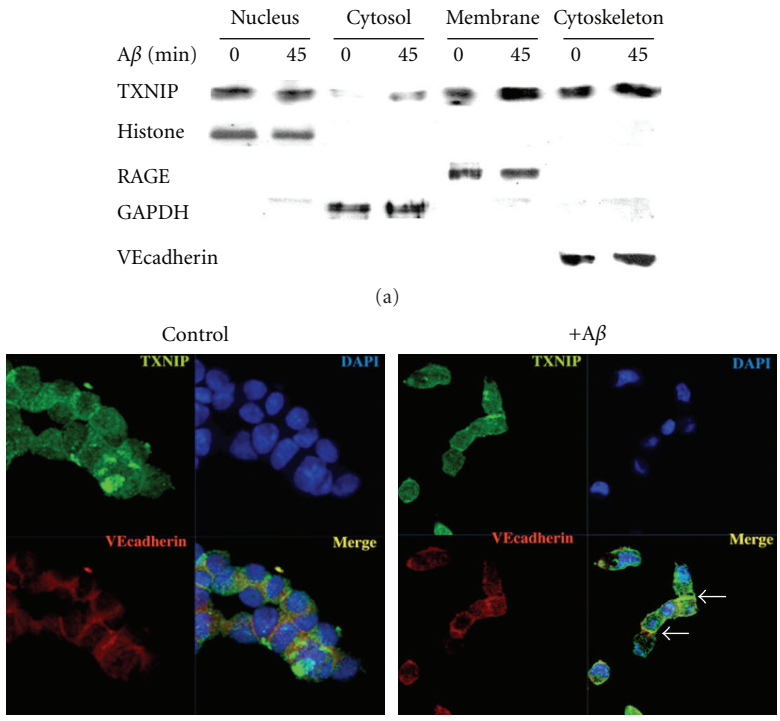


Figure 3: Aβ enhances TXNIP translocation to the plasma membrane. (a) RBE4 cells were maintained 5 days in differentiation medium (F10/MEM, 2.5% FCS, hydrocortisone 14 μM, Hepes 10 mM, bFGF 1 μg/mL). RBE4 cells were stimulated for 45 min with Aβ1-42 (3 μM). Subcellular fractions were obtained using a cell fractionation kit (Biorad) according to the manufacturer instruction. The presence of TXNIP, RAGE, VE-cadherin, and histone H3 were analyzed by western blotting. (b) RBE4 cells were maintained as described in (a) and stimulated for 45 min with Aβ1-42 (3 μM). Cells were fixed in PBS containing 4% PFA and permeabilized 10 min in PBS 0.1% Triton X-100. Cells were maintained 1 h in blocking solution (PBS 3% BSA) at room temperature and then incubated over/night at 4°C with a rabbit anti-TXNIP polyclonal antibody (Invitrogen) and a mouse anti-VEcadherin monoclonal antibody (Santa Cruz biotechnology) in blocking solution. Cells were washed 3 times for 15 min with PBS and incubated with the appropriate secondary antibody. Nuclei were stained with Hoecst. Immunofluorescence was analyzed by a confocal microscopy (Zeiss). These data are representative of 3 independent experiments.

showed that patient, who had received the low dose of PF-04494700 showed an improved ADAS-cog score after 18 months, when compared to the placebo group, even if they were taken off the treatment with PF-04494700 after 12 months. Thus, Galasko suggests that it was an error to stop the clinical trial, at least with the low-dose group. The researcher also reported that the high-dose group completely recovered with the ADAS-cog score after 18 month; thus, the toxic effect was reversible. He did not explain the reason of the toxicity induced by the higher dose of PF-04494700. As outlined in the present, RAGE participates in neurite outgrowth, and RAGE is highly expressed in brain neurons during the development. The higher dose of PF-04494700 might thus block or at least interfere with the yet mot clearly defined physiological functions of RAGE, thereby affecting neurogenesis. On the contrary, the lower dose of PF-04494700, which was beneficial in the long time, suggests that the inhibition of chronic RAGE activation can ameliorate AD progression and imply follow-up studies using low dose of PF-04494700 to inhibit RAGE-induced chronic neurovascular dysfunction.

## 8. Conclusions and Hypothesis

Herein, we summarize all studies indicating that RAGE participates in sporadic AD progression by activating several pathways in different cell types, particularly BBB, glia, and neurons (Figure 4). These pathways converge and ultimately lead to synaptic dysfunction and neurodegeneration. We also report ongoing studies demonstrating that RAGE participates in AD progression by inducing TXNIP expression. We previously demonstrated that RAGE-TXNIP axis is induced in different cell types and promotes inflammation [26, 27]. Moreover, we have shown that enhanced TXNIP expression in diabetes ultimately leads to neurodegeneration [30]. In the present paper, we show that RAGE-TXNIP axis is induced in brain endothelial cells. In addition, we demonstrate for the first time that TXNIP is early overexpressed in the hippocampus of an AD mouse model. Several studies suggest that brain insulin resistance is implicated in AD progression. However, the molecular mechanisms leading to brain insulin resistance in AD are still unknown. Our data are suggesting that RAGE may induce brain insulin resistance by enhancing TXNIP expression. Only one study demonstrated that RAGE triggering induces insulin resistance and impairs glucose uptake in skeletal muscle [102]. Induction of RAGE-TXNIP axis in AD brain can further demonstrate the role of RAGE in amplifying age-induced oxidative stress. Indeed, TXNIP induces oxidative stress. The analysis of A$\beta$-induced TXNIP expression in glial and neuronal cells is under investigation. However, we and other demonstrated that TXNIP is necessary to induce IL-1$\beta$ expression [27, 103] and to promote neurodegeneration [30, 93]. Thus, we hypothesize that RAGE-TXNIP axis participates in AD progression by activating a concerted action of oxidative stress, inflammation, vascular dysfunction, and neurodegeneration.

We also hypothesize that pharmacological treatments aimed to inhibit chronic RAGE activation will be beneficial in blocking neurovascular dysfunction in AD, thereby conferring a neuroprotective effect by restoring the physiological function of RAGE and TXNIP that are implicated in neuronal differentiation and repair. Thus, a prolonged treatment with a low dose of PF-04494700 might block the effects induced by RAGE chronic activation and ameliorate AD progression.

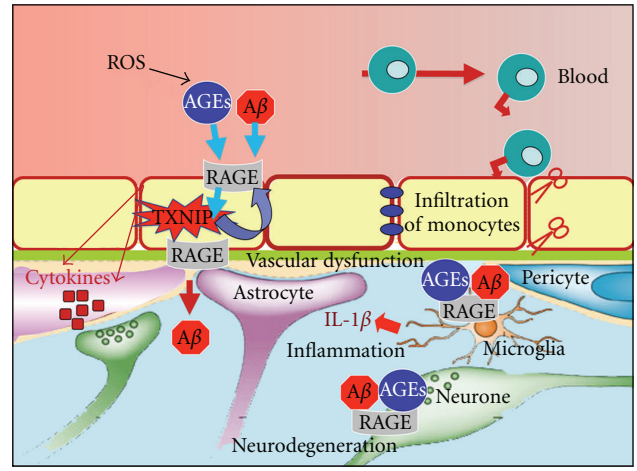


Figure 4: PF-04494700, an inhibitor of RAGE, can ameliorate sporadic AD and promote neuroprotection by blocking RAGE activation in various cell type. Aging-induced oxidative stress leads to the formation of AGEs, which activate RAGE together with A$\beta$ in various cell type. Triggering of RAGE at the BBB leads to TXNIP expression and subsequent inflammation, BBB leakage, and monocytes infiltration. Moreover, RAGE triggering induces a positive feedback loop enhancing RAGE expression, resulting in enhanced transport of A$\beta$ from the blood to the brain. RAGE activation in glial cells promotes proinflammatory gene expression, which enhanced A$\beta$ production inside the brain and neurotoxicity. RAGE triggering in neuronal cells induces oxidative stress and the production of M-CSF, leading to inflammation. Thus, activation of RAGE in different cell types orchestrates the neuroinflammatory processes that ultimately lead to neurodegeneration. Thus, treatments aimed to inhibit chronic RAGE activation will confer a neuroprotective effect by blocking RAGE-mediated neurovascular dysfunction.

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