THE METABOLIC-INFLAMMATORY AXIS IN BRAIN AGING AND NEURODEGENERATION

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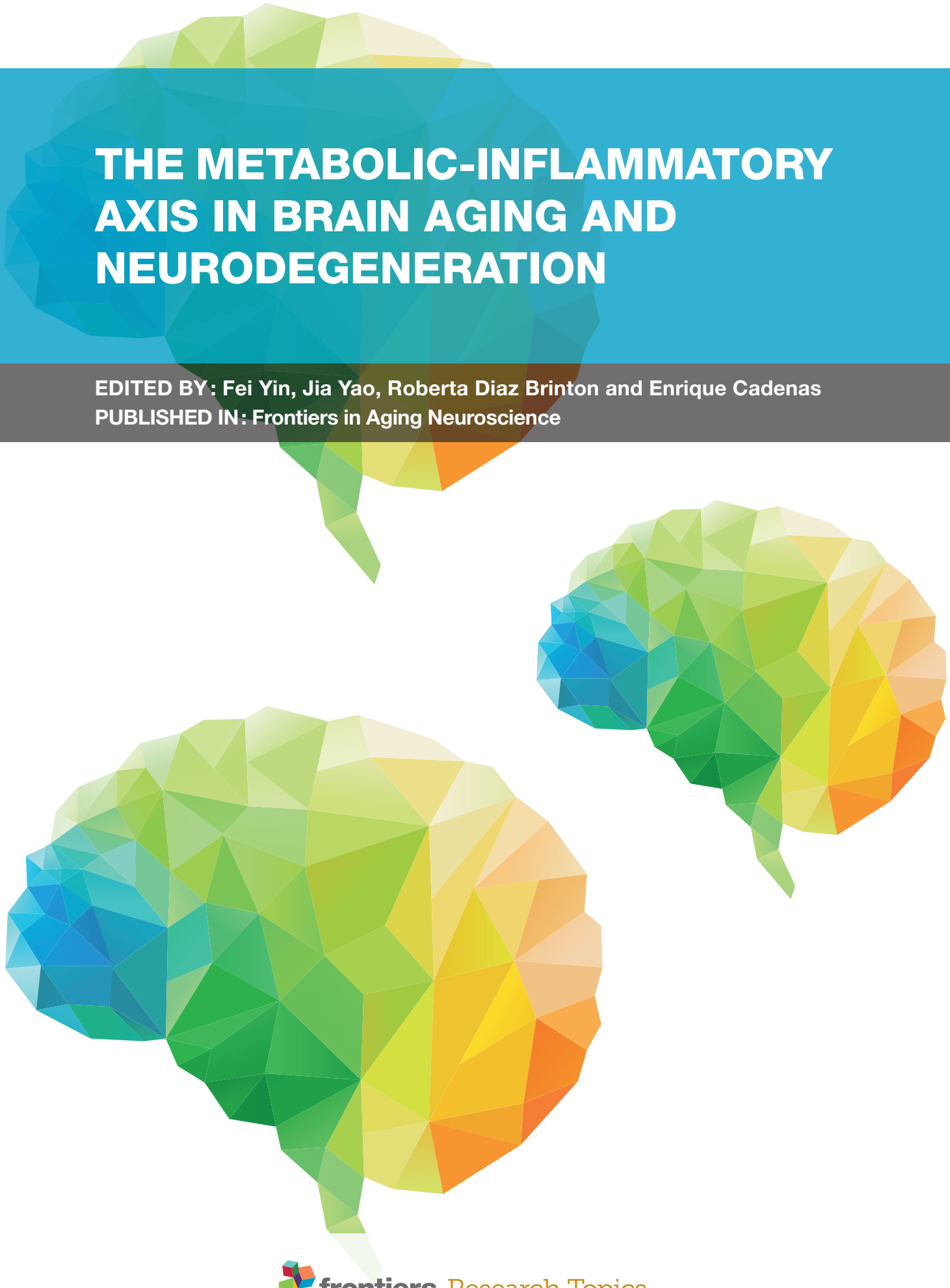

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THE METABOLIC-INFLAMMATORY AXIS IN BRAIN AGING AND NEURODEGENERATION

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Impairment of energy metabolism is a hallmark of brain aging and several neurodegenerative diseases, such as the Alzheimer's disease (AD). Age- and disease-related hypometabolism is commonly associated with oxidative stress and they are both regarded as major contributors to the decline in synaptic plasticity and cognition. Neuroinflammatory changes, entailing microglial activation and elevated expression of inflammatory cytokines, also correlate with age-related cognitive decline. It is still under debate whether the mitochondrial dysfunction-induced metabolic deficits or the microglia activation-mediated neuroinflammation is the initiator of the cognitive changes in aging and AD. Nevertheless, multiple lines of evidence support the notion that mitochondrial dysfunction and chronic inflammation exacerbate each other, and these mechanistic diversities have cellular redox dysregulation as a common denominator.

This research topic focuses on the role of a metabolic-inflammatory axis encompassing the bioenergetic activity, brain inflammatory responses and their redox regulation in healthy brain aging and neurodegenerative diseases. Dynamic interactions among these systems are reviewed in terms of their causative or in-tandem occurrence and how the systemic environment, –e.g., insulin resistance, diabetes, and systemic inflammation–, impacts on brain function.

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EDITORIAL
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Editorial: The Metabolic-Inflammatory Axis in Brain Aging and Neurodegeneration

Fei Yin[1]*, *Jia Yao*[1], *Roberta D. Brinton*[1,2,3] *and Enrique Cadenas*[1]

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Keywords: brain metabolism, redox homeostasis, Alzheimer's disease, neuroinflammation, mitochondria

Editorial on the Research Topic

The Metabolic-Inflammatory Axis in Brain Aging and Neurodegeneration

INTRODUCTION

Impairment of energy metabolism is a hallmark of brain aging and several neurodegenerative diseases, such as the Alzheimer's disease (AD). Age- and disease-related hypometabolism is commonly associated with oxidative stress and they are both regarded as major contributors to the decline in synaptic plasticity and cognition. Neuroinflammatory changes, entailing microglial activation and elevated expression of inflammatory cytokines, also correlate with age-related cognitive decline. It is still under debate whether the mitochondrial dysfunction-induced metabolic deficits or the microglia activation-mediated neuroinflammation is the initiator of the cognitive changes in aging and AD. Nevertheless, multiple lines of evidence support the notion that mitochondrial dysfunction and chronic inflammation exacerbate each other, and these mechanistic diversities have cellular redox dysregulation as a common denominator.

This research topic focuses on the role of a metabolic-inflammatory axis (Yin et al., 2016) encompassing the bioenergetic activity, brain inflammatory responses, and their redox regulation in healthy brain aging and neurodegenerative diseases. Dynamic interactions among these systems are reviewed in terms of their causative or in-tandem occurrence and how the systemic environment,—e.g., insulin resistance, diabetes, and systemic inflammation—, impacts on brain function.

ENERGY METABOLISM IN BRAIN AGING AND NEURODEGENERATION

Brain energy metabolism starts with the uptake of energy fuels—primarily glucose—from the cerebrovasculature. In the review contributed by Lourenço et al., the derailment of neurovascular and neurometabolic functions was discussed in both normal aging and AD, with the nitric oxide (·NO)-mediated neurovascular coupling being proposed as a key factor implicated in the progression of neuronal dysfunction.

In addition to glycolysis, glucose in the brain is metabolized via the pentose phosphate pathway (PPP) to generate NADPH—the major reducing equivalent in the cell supporting GSH- and thioredoxin-dependent antioxidant systems (Yin et al., 2014). Bouzier-Sore and Bolaños suggested that age- and disease-related decline in glucose uptake is responsible for not only the reduced

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bioenergetic capacity but also the neuronal oxidative damage, inasmuch as the contribution of PPP to neuronal glucose metabolism is underestimated with current assessment approach.

Following their uptake into the brain and subsequent cytosolic reactions, energy fuels are primarily metabolized in mitochondria—the only organelles that possess their own genome (mtDNA). Toward this unique property, Wang and Brinton comprehensively discussed the advances in the field in support of mitochondrial genetic variance/haplotypes as another important genetic risk factor for AD, together with APOE4 and chromosomal sex. Moreover, a precision medicine approach was proposed to incorporate these major risk factors into the design and development of future therapeutics for late onset AD.

Regulation of mitochondrial function is coordinated by both mtDNA and the nuclear genome, with the latter encoding ∼99% of the proteins of these organelles. To maintain an efficient, dynamic, and responsive mitochondrial network, a complex multi-layer quality control system is utilized by the cell (Yin and Cadenas, 2015). Recently, the field of mitochondria biology has been focused on the dynamic and interactive features of these semi-autonomous organelles and a complex multi-layer quality control system coordinating their function with environmental changes. In this regard, Zorzano and Claret reviewed the vital role of mitochondrial dynamics in the pathogenesis of neurodegenerative diseases, as well as in the hypothalamic dysfunction that is implicated in imbalanced energy metabolism.

REDOX REGULATION CONNECTS BRAIN METABOLISM AND NEUROINFLAMMATION

The aging- and neurodegenerative brains are found to be associated with a mild, chronic neuroinflammation primarily due to dysregulated innate immunity, with microglia senescence playing a central role. In the review by von Bernhardi et al., the key elements and pathways of microglia action and their potential as a therapeutic target in brain aging and AD were discussed comprehensively. It was proposed that microglia aging and its mal-activation, induced by mitochondrion- and non-mitochondrion-derived oxidative stress and endolysosomal dysfunction, initiate and further exacerbate neuroinflammation, resulting in synaptic dysfunction, and eventually neurodegeneration.

It is well-known that deficits in metabolic and mitochondrial function lead to the impairment of redox homeostasis, which is one of the major stimulators of the neuroinflammatory responses in aging and AD. The review by Gamba et al. suggested that altered cholesterol metabolism (and hypercholesterolemia) mediates such a connection and thereby contributes to AD progression, via a vicious cycle involving redox perturbation, oxidized cholesterol, neuroinflammation, Aβ load, and neuronal damage.

Further, in the review contributed by De Felice and Lourenco, brain metabolic dysregulation in AD was expanded from glucose metabolism to Aβ oligomer (AβO)-induced neuroinflammation and ER stress (unfolded protein response, UPR), with the latter two processes impacting not only synaptic and cognitive functions, but also peripheral insulin resistance, glucose intolerance, and diabetes. It was also suggested that UPR activation might be a common pathogenic mechanism shared by multiple neurological disorders.

PERIPHERAL- AND ENDOCRINE INTERACTIONS WITH THE BRAIN METABOLIC-INFLAMMATORY AXIS

The connection between brain function and the systemic environment is bidirectional, as indicated by the important role of peripheral metabolism in the progression of brain aging and neurodegeneration. In this Research Topic, Christensen and Pike discussed the role of obesity and systemic inflammation in AD pathogenesis with a special emphasis on women, who have a two-fold increased AD risk compared to men. In addition to the obesity-induced inflammation, increased AD risk in females also correlated with the elevated inflammation due to estrogen depletion upon perimenopause and menopause. The authors thereby proposed an interactive set of risk factors implicated in AD, including aging, menopause, adiposity, and inflammation.

Like adiposity, the risk of type 2 diabetes (T2D) for AD was also mediated through low-grade inflammation. In the report by Gorska-Ciebiada et al. in a group of T2D patients, it was found that serum levels of inflammatory parameters, such as C-reactive protein, advanced glycation end products (AGE), and their receptors, were increased only in those with mild cognitive impairment (MCI), a critical transitional stage between normal cognitive aging and AD.

Brain function and neurodegenerative risks are not only affected by the systemic metabolic status, but also the endocrine system. As reported by Morgan and Finch, the onset of perimenopause increased the estrogen receptor-alpha to estrogen receptor-beta ratio (ERα/ERβ) in astrocytes, which was associated with impaired neurotrophic responses to estradiol. These endocrine-related changes occurred during female perimenopausal transition could thus be implicated in the increased risk for AD in women (Brinton et al., 2015).

The review contributed by Paul et al., focused on the role of glucocorticoids—another family of anti-inflammatory steroid hormones—and their receptors in the context of stress-induced cognitive decline in aging and AD. The interactive molecular links of glucocorticoids with the cholinergic system and the hypothalamic-pituitary-adrenal (HPA) axis were proposed as a contributing factor to cognitive impairment and a target of pharmacotherapeutic strategies.

Two potential neuroprotective strategies targeting the metabolic or inflammatory pathways were discussed in this topic. In a study utilizing an early stage Parkinson's disease animal model, Farmer et al. suggested that immune system signaling molecules, granulocyte macrophage-colony stimulating factor (GM-CSF) and erythropoietin (EPO), exhibited neurotrophic and neuroprotective capabilities to re-innervate striatal neurons. In terms of nutraceutical strategies targeting brain oxidative and inflammatory pathways, Ong et al. contributed a literature

review of the neuroprotective mechanisms and potential of ginsenosides, the bioactive ingredients in ginseng root, against multiple neurodegenerative disorders.

PERSPECTIVES

Brain aging and neurodegenerative diseases have a multifactorial nature. For example, AD is characterized by not only the accumulation of the Aβ oligomers and fibrils, but also metabolic and inflammation changes such as glucose hypometabolism, blood-brain-barrier (BBB) disruption and glial activation. Recent failures of multiple clinical trials targeting Aβ continue to fuel the debate on the Aβ-centric view of AD pathogenesis. We hope this research topic has created a forum for in-depth discussions that help evolve the sequential view of the mechanisms inherent in brain aging and progression of neurodegeneration to the understanding that several mechanisms—centered on a dynamically coordinated mitochondrial network and the metabolic-inflammatory axis—co-exist and interact with each other with significant contribution from the systemic environment such as insulin resistance and peripheral inflammation.

AUTHOR CONTRIBUTIONS

FY drafted the manuscript that was reviewed and edited by JY, RDB, and EC. All authors co-edited the Research Topic.

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MINI REVIEW
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Neurovascular and neurometabolic derailment in aging and Alzheimer's disease

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The functional and structural integrity of the brain requires local adjustment of blood flow and regulated delivery of metabolic substrates to meet the metabolic demands imposed by neuronal activation. This process—neurovascular coupling—and ensued alterations of glucose and oxygen metabolism—neurometabolic coupling—are accomplished by concerted communication between neural and vascular cells. Evidence suggests that neuronal-derived nitric oxide (•NO) is a key player in both phenomena. Alterations in the mechanisms underlying the intimate communication between neural cells and vessels ultimately lead to neuronal dysfunction. Both neurovascular and neurometabolic coupling are perturbed during brain aging and in age-related neuropathologies in close association with cognitive decline. However, despite decades of intense investigation, many aspects remain poorly understood, such as the impact of these alterations. In this review, we address neurovascular and neurometabolic derailment in aging and Alzheimer's disease (AD), discussing its significance in connection with •NO-related pathways.

Keywords: neurovascular coupling, neurometabolism, nitric oxide, Alzheimer's disease, aging

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Introduction

The proper function of the brain depends critically upon constant and regulated blood supply. Despite representing only 2% of total body mass in the adult human, the brain is an energy expensive organ, consuming *circa* one fifth of the available oxygen and glucose (Zlokovic, 2011). Upon neural excitation, local metabolic rate may increase as much as 50% relative to basal values depending on the intensity of stimulation (Shulman and Rothman, 1998). Paradoxically, the intrinsic energy reserves are minimal (Kealy et al., 2013), which implies that, to assure an appropriate balance, changes in blood supply must be attuned to the physiological demands imposed by neural activation with high temporal and regional precision. The accomplishment of such interplay depends on the complex and concerted communication between neurons, astrocytes, pericytes, microglia, and vascular cells. Active neurons generate signals that are transduced at blood vessels to locally adjust blood flow and guarantee efficient delivery of bioenergetic substrates—a process termed neurovascular coupling. Furthermore, the profile of neuronal activity is closely associated with glucose and oxygen metabolism—neurometabolic coupling.

Neurovascular coupling has been a matter of intense investigation over the last decades and is yet not fully understood. Nonetheless, there are generally accepted propositions, such as (1) the process relies on glutamate-dependent pathways, in a feed-forward mechanism; (2) likely several molecules and/or pathways cooperate to translate the need for substrates imposed by the neuronal

activity into changes in cerebral blood flow, and (3) the underlying mechanisms can be distinct throughout the brain areas, reflecting specificities of the neuronal networks. Amongst several molecules proposed to mediate neurovascular coupling, nitric oxide ($^{\bullet}$NO), a free radical intercellular messenger, has emerged as an attractive candidate (Iadecola, 1993). In the brain, $^{\bullet}$NO is produced upon glutamatergic activation by the neuronal isoform of nitric oxide synthase (nNOS), which is physically anchored and functionally coupled to the NMDA-type glutamate receptor (Christopherson et al., 1999). Nitric oxide is endowed with peculiar physicochemical properties (radical nature, small size, diffusibility, hydrophobicity), that determine a diversity of biological targets and pathways in which it is involved (for review Winkler and Luer, 1998; Guix et al., 2005).

Despite some inconsistent observations, ample evidence suggests that $^{\bullet}$NO plays a critical role in neurovascular coupling, particularly in the hippocampus and cerebellum (Rancillac et al., 2006; Lourenço et al., 2014a). Direct and *in vivo* data show that $^{\bullet}$NO is a direct mediator of the process, bridging neurons and blood vessels. The simultaneous monitoring of $^{\bullet}$NO fluctuations and CBF changes during glutamatergic activation, coupled to pharmacological approaches, strengthens the notion that $^{\bullet}$NO produced by neurons can diffuse toward neighboring blood vessels and promote vasodilation via activation of soluble guanylate cyclase (sGC). In turn, the involvement of endothelial-derived $^{\bullet}$NO appears to be negligible (Lourenço et al., 2014a). In addition to participating in neuron-to-blood vessel signaling pathways, $^{\bullet}$NO may be involved in the regulation of ensued processes, such as neurometabolic coupling. Nitric oxide can regulate energy metabolism/cellular respiration by interfering with several signaling pathways. For instance, at low nM concentrations $^{\bullet}$NO regulates mitochondrial respiration by inhibiting cytochrome c oxidase (CcO) in competition with O_2 (Rossignol et al., 2003; Moncada and Bolaños, 2006; Antunes and Cadenas, 2007). This competitive process allows not only for the fine tuning of mitochondrial respiration, but may also facilitate O_2 distribution from the microvasculature to sites of up-regulated energetic demand (Giulivi, 2003; Victor et al., 2009) or modulate production of mitochondrial-derived signaling molecules such as superoxide and hydrogen peroxide (Cadenas, 2004). Nonetheless, when $^{\bullet}$NO fluxes are increased in biological systems in concurrence with an unbalanced redox environment, production of $^{\bullet}$NO-derived reactive species such as peroxynitrite can significantly perturb mitochondrial function by inhibiting complex I of the mitochondrial respiratory chain, aconitase and Mn-superoxide dismutase (Brown, 2007). This Dr. Jekyll and Mr. Hyde type of bioactivity can also be observed for glycolysis, where $^{\bullet}$NO has been shown to boost glycolytic turnover in a cGMP dependent mechanism in astrocytes (but not neurons) (Bolanos et al., 2008) while gluteraldehyde-3-phosphate dehydrogenase can be inhibited by nitration (Palamalai and Miyagi, 2010).

Interestingly, the impact of $^{\bullet}$NO on brain metabolic status can reflect upon neurovascular coupling. Variations in O_2 concentration can alter vascular tone, both by affecting the synthesis of the vasoactive messengers (including $^{\bullet}$NO itself), and by altering the levels of lactate and adenosine, which modulate pathways underlying neurovascular coupling (Gordon et al., 2008; Attwell et al., 2010).

In sum, neuronal activity produces $^{\bullet}$NO, a messenger that diffuses to blood vessels inducing vasodilation. Consequently, increased delivery of energy substrates impacts local neuronal metabolism and function. Therefore, the two processes, neuronal activity-dependent CBF increase and oxygen and glucose utilization by active neural cells are inextricably linked, establishing a functional metabolic axis in brain, the neurovascular-neuroenergetic coupling axis. Nitric oxide is a master regulator of this axis. The perturbation of this $^{\bullet}$NO-driven regulatory cycle can trigger a sequence of events that may ultimately lead to neuronal dysfunction. Ample evidence supports the notion that alterations of the regulatory mechanisms involved in neurovascular and neurometabolic coupling lead to neuronal dysfunction and disease, as discussed in the following sections.

Dysfunction in Neurovascular Coupling During Brain Aging and Alzheimer's Disease

It is increasingly accepted that brain aging and age-associated diseases, such as Alzheimer's disease (AD), share some common histological and pathophysiological alterations that, ultimately, underlie compromised cognitive status. The possible contribution of abnormal cerebrovascular function to progressive functional decline has been vigorously emphasized and is nowadays well recognized. However, the putative triggers of this dysfunction remain a matter of extensive debate (Zlokovic, 2011; Kalaria, 2012; de la Torre, 2012). Age is the most relevant risk factor for the sporadic form of AD, the leading cause of dementia in the elderly. Although AD results predominantly from neurodegenerative changes, there is a growingly recognized contribution of well-defined decline in cerebrovascular parameters (Girouard and Iadecola, 2006). Between 60 and 90% of AD patients exhibit cerebrovascular pathologies including cerebral amyloid angiopathy, microinfarcts and ischemic lesions, blood–brain barrier disruption,and microvascular degeneration (Jellinger and Mitter-Ferstl, 2003; Bell and Zlokovic, 2009). Converging on this idea, the ethiopathogenetic role of a spectrum of chronic vascular disorders such as hypertension, hypercholesterolemia and type 2 diabetes has been proven to be present in the pathogenesis of AD (Kalaria, 2012).

The alterations in cerebrovascular function in aging and AD can be reflected by both chronic brain hypoperfusion and altered neurovascular coupling. Numerous clinical studies, based on evaluation of resting CBF in human subjects, unanimously recognize a negative correlation between global CBF and age (Krejza et al., 1999; Schultz et al., 1999; Fisher et al., 2013; Fabiani et al., 2014). Also, several lines of evidence support cerebral hypoperfusion as a preclinical condition in AD and one of the most accurate predictors for developing AD. Studies of brain function during behavioral tasks suggest age-related differences in activation, as well as differences between patients with AD and age-matched control subjects, revealing a close correlation with

cognitive decline (Alsop et al., 2000; Ruitenberg et al., 2005; Xu et al., 2007). A significant observation is that the BOLD signal evaluated by fMRI during an associative encoding task is reduced in individuals carrying the APOE-ε4 allele, a recognized risk factor for sporadic AD (Fleisher et al., 2009).

However, whereas the correlation between cerebrovascular changes and cognitive decline has been firmly established, the neurobiological link between the two is still poorly defined, as is the causality (Iadecola, 2004). Evidence for cerebrovascular changes resulting in neuronal damage, hastening neurodegeneration, is clear but it is also known that neurogenic factors can underlie cerebrovascular dysfunction. Amyloid β peptide (Aβ), the main component of the amyloid plaques found in AD, may play a major role in cerebrovascular impairment, as it is known to disrupt the physiological mechanisms regulating CBF. Elevations of Aβ levels in rodent models of AD are associated with lower resting CBF and impaired vasodilatory responses in cerebral circulation (Iadecola et al., 1999; Niwa et al., 2002), including neurovascular coupling (Niwa et al., 2000a). Additionally, Aβ peptides can impair endothelium-dependent relaxation and enhance vasoconstriction (Thomas et al., 1996; Niwa et al., 2000b, 2001) by either promoting oxidative stress in the vascular cells (Hamel et al., 2008) and/or inhibiting the production of neuronal-derived vasodilating messengers (Iadecola, 2004) such as •NO (Venturini et al., 2002).

In an attempt to help clarify this controversy, we have observed age-dependent impairment of •NO-dependent neurovascular coupling both in rodent models of AD and aging. Furthermore, we found that this dysfunction appears to be primarily of cerebrovascular rather than neuronal origin. These studies were carried out in a triple transgenic mouse model of AD (3xTg-AD mice) and in Fisher 344 rats (widely used in brain aging studies). By simultaneously measuring •NO and CBF in the hippocampus, we observed that glutamate-evoked increase in CBF was diminished during aging and AD, despite the fact that •NO signaling remains almost unaltered. The effect of aging/AD on •NO-dependent CBF changes is summarized in **Figure 1**. Data obtained in 3xTg-AD mice revealed a shift in the CBF changes coupled to the •NO temporal dynamic elicited by glutamatergic activation. This is reflected by the increased delay on the onset of CBF increase (**Figure 1C**), as well as by the decrease in the amplitude of CBF change. The later leads to the abolishment of the correlation between •NO and CBF observed in control animals (**Figure 1B**). Similar observations were obtained during aging in F344 rats, with CBF changes declining 39% from 6 to 12 months, and a further 36% from 12 to 23 months, with no significant decrease in •NO signaling (unpublished data). Of note, the deterioration of the neurovascular coupling in both cases preceded an obvious impairment in cognitive function as accessed by behavior tests.

Overall, these results strengthen the notion of cerebrovascular dysfunction as a fundamental process underlying AD pathophysiology and brain aging. That is, while neuronal •NO signaling remains functional it is not conveniently transduced into vasodilation at blood vessels. Amongst the potential causes, changes of the redox environment in blood vessels may result in the quenching of •NO, impeding it's binding to sGC. In fact,

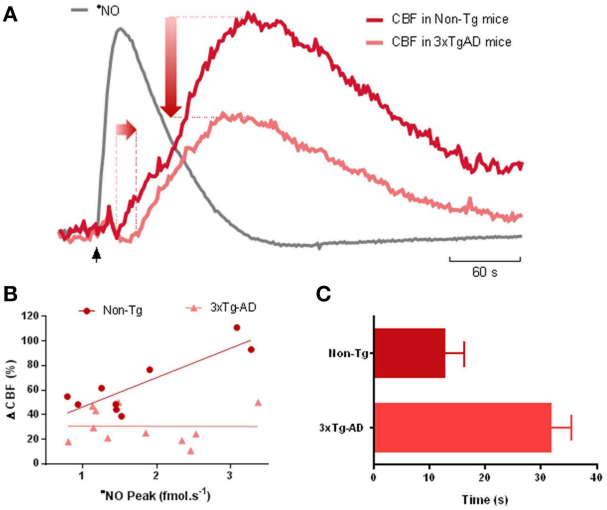

FIGURE 1 | Impairment of nitric oxide-dependent neurovascular coupling in AD. (A) Representative recordings of the simultaneous measurements of •NO concentration dynamics and CBF changes in the hippocampus of 12 months-old Non-Tg mice and 3xTg-AD mice in response to L-glutamate. L-glutamate (0.5 nmol, 1 s) was locally applied at time indicated by the black vertical arrow. The measurements were performed using •NO selective microelectrodes and Laser Doppler flowmetry as previously described (Lourenço et al., 2014a). The temporal dynamic of •NO is roughly identical in both strains and thus only a representative trace is presented (gray line). The CBF change coupled to the transient •NO increase showed a temporal delay and decrease in amplitude in 3xTg-AD mice (light red line) as compared to Non-Tg mice (dark red line). **(B)** The linear relationship between •NO peak amplitude and the amplitude CBF change observed in Non-Tg mice ($p = 0.003$) was lost in 3xTg-AD mice ($p = 0.981$). In the former, the linear regression showed an $R^2 = 0.735$ and a slope of 24% CBF/fmol s^{-1} •NO. **(C)** Delay between the onset of •NO transient and the onset of the CBF change in 12-months old 3xTg-AD mice and Non-Tg mice. Values represent the mean ± SEM ($p = 0.001$).

previous reports show that vascular oxidative stress may have serious implications in cerebrovascular function (reviewed in Hamel et al., 2008).

A deep understanding of the underlying mechanisms by which altered cerebrovascular function influences AD neuropathology has not yet been achieved. Nevertheless, in accordance with the hypothesis prompted by the vascular-driven theory of AD, cerebrovascular dysfunction and consequent hypoperfusion is suggested to be linked to (1) enhanced β-secretase protein expression, which potentiates Aβ overproduction and altered phosphorylated tau (Velliquette et al., 2005; Koike et al., 2010); (2) faulty clearance of Aβ peptides, favoring accumulation in the brain (Girouard and Iadecola, 2006) and (3) reduced supply of metabolic substrates and neurometabolic dysfunction.

Altered Neurometabolism in Brain Aging and Alzheimer's Disease

Brain aging is accompanied by widespread metabolic alterations associated with cognitive decline that connect with the so called "metabolic syndrome" (Barzilai et al., 2012).

Components of metabolic syndrome such as insulin resistance or hypercholesterolemia are predictors of accelerated cognitive decline and dementia, particularly AD (Haralampos et al., 2008). The pathways linking these metabolic alterations and the decay of cognitive function are still poorly understood, but mitochondrial dysfunction has been identified as a link between the two. Besides changes in neurotransmission processes (decrease in glutamate and GABA), brain aging and AD are associated with perturbations of primary energy metabolism (use of glucose and lactate) as well as in the turnover of lipid membranes (Castegna et al., 2004; Mohmmad Abdul and Butterfield, 2005; Bader Lange et al., 2008; Duarte et al., 2014). Mitochondria play an essential role in cellular respiration and are responsible not only for the production of ATP, but also for Ca^{2+} buffering, production and removal of reactive oxygen species, as well as of signaling molecules that regulate cell cycle, proliferation and apoptosis (for review Yin et al., 2014). The brain's consumption of glucose is primarily driven by the constant need to maintain ionic gradients in pre- and post-synaptic compartments in order to sustain excitability, as well as to maintain transmembrane lipid asymmetries (Harris et al., 2012). Considering the high energetic demand of this organ and it's excitability requirements, deregulation of optimal mitochondrial performance can significantly impact neurometabolism and function.

During the past decades, intensive research has shown that brain energy metabolism in impaired during the progression of AD (de Leon et al., 1983; Mosconi et al., 2005; Reiman et al., 2005; Scholl et al., 2011; Yin et al., 2014). Amyloidosis, or more specifically plaque pathology (Gearing et al., 1995), although present in roughly 90% of AD patients, is not in itself sufficient to account for the disease and many authors have reported that amyloid load may not necessarily correlate with dementia (Gearing et al., 1995; Hsia et al., 1999; Mucke et al., 2000; Swerdlow et al., 2014). As the amyloid cascade hypothesis of AD has evolved over the years, the concept of plaque as the causal factor of disease has given way to the notion that soluble forms of Aβ oligomers are in fact the toxic moiety (Lesne and Kotilinek, 2005; Walsh et al., 2005; Lesne et al., 2006). These oligomers have been shown to compromise the function of organelles such as the mitochondria.

Thus, the concept that mitochondrial dysfunction is a key contributor to the onset and progression of AD has become firmly consolidated and the "mitochondrial cascade hypothesis" has gained significant ground, especially with regards to late-onset AD (Chaturvedi and Flint Beal, 2013; Swerdlow et al., 2014). This hypothesis is sustained not only on the fact that both Aβ and hyperphosphorylated tau are capable of altering mitochondrial function, but also takes into account the major risk factor for sporadic AD—age. One important consequence of biological aging is the accumulation of somatic mtDNA mutations, which contribute to physiological decline and neurodegenerative disease (Lin et al., 2002). The impact of these point mutations on each individual as they age will be influenced by inherited and environmental factors (Wallace, 1992; Swerdlow et al., 2014).

Studies in both animal models and AD patients have shown that Aβ is capable of inhibiting the complexes of the mitochondrial respiratory chain, as well as the TCA cycle enzyme α-ketogluterate dehydrogenase (Casley et al., 2002; Manczak et al., 2006). Impairment of oxidative phosphorylation (OxPhos) is mainly due to the inhibition or decreased activity of CcO, which, along with ATP synthase, is known to be oxidized in the brains of AD patients (Kish et al., 1992; Mutisya et al., 1994; Maurer et al., 2000). Furthermore, mitochondrial dysfunction promotes tangle formation in AD by contributing to tau phosphorylation (Melov et al., 2007).

Using high resolution respirometry for evaluation of O_2 consumption rates (OCR) in intact hippocampal slices obtained from 3xTg-AD mice and age-matched controls (**Figure 2A**) we have observed an age-dependent impairment of OxPhos in old-aged animals which tends to be more evident in old-aged 3xTg-AD (**Figure 2B**). Both basal and maximal O_2 consumption rates decrease as a function of age in both genotypes. More relevantly, the sparing capacity has diminished in both old-age groups, implying a lower capacity to respond to energy-demanding situations (such as increased excitability) with adequate increase OxPhos to supply ATP. Decrease in basal tissue OCR was further revealed when we determined the drop in $[O_2]$ from the surrounding media to the tissue core (**Figure 2C**), which was significantly smaller in old-age 3TgAD animals. This drop in $[O_2]$ is expected to positively correlate with the metabolic activity state of the tissue. Of note is the fact that this 3xTg-AD model, although expressing a progressive AD phenotype as expected to occur in humans (Oddo et al., 2003), is a model of familiar AD and not sporadic AD. As such, it is not surprising to us to find that the major contributor to changes in OxPhos is actually age and not genotype. Sporadic AD is not associated with deterministic gene mutations, although some genetic influences have been identified, such as the APOE-ε4 variant (Corder et al., 1993; Swerdlow, 2007).

Hypometabolism in AD is most likely more than a mere consequence of the cellular and functional degeneration (decreased brain function would obviously require less energy substrate supply)—recent observations suggest that optimal glucose utilization is impaired in early asymptomatic stages of the disease and may contribute or precipitate AD neuropathology. Data collected from both clinical and animal model research strongly suggest that significant decrease in brain metabolism occurs well before any clinical manifestation of AD, namely measurable cognitive decline (for review Petrella et al., 2003).

Changes in the concentration dynamics of $^{\bullet}NO$ in the brain may contribute to altered neurometabolism in aging and AD. In structures of the CNS intimately linked to memory and learning, such as the hippocampus, $^{\bullet}NO$ is produced by neurons upon activation of glutamatergic synapses (Ledo et al., 2005, 2015; Lourenço et al., 2011, 2014b). Besides its obvious role as a neuromodulator, acting namely as a retrograde messenger in plasticity phenomena associated with memory and learning (Prast and Philippu, 2001), $^{\bullet}NO$ has been looked upon as a master regulator of neurometabolism, as discussed above. Alongside or as a consequence of inhibition of the mitochondrial electron transporting chain (Antunes and Cadenas, 2007), $^{\bullet}NO$ has also been shown to boost glycolytic rate and glucose uptake (reviewed in Almeida et al., 2005). One can hypothesize that

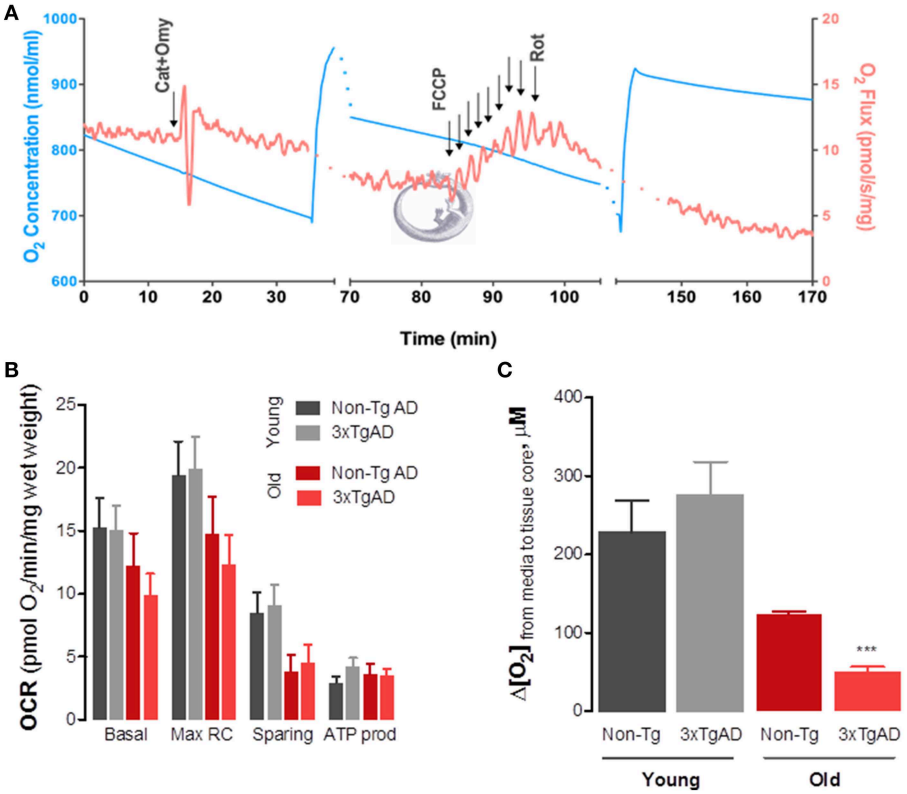

FIGURE 2 | Changes in mitochondrial oxidative phosphorylation in intact hippocampal slices from 3xTg-AD mice and Non-Tg mice show significant effect of aging on basal and maximal respiratory rates as well as sparing capacity. We developed a protocol that enabled us to evaluate OxPhos in intact hippocampal slices obtained from young and old-aged mice. Using a high-resolution respirometer (Oxygraph-2K, by Oroboros Instruments, Austria) we determined O_2 consumption rates (OCR) or O_2 flux (red line in **A**). Due to high O_2 requirement of hippocampal slices, experiments were performed at high [O_2] and chambers were re-oxygenated throughout the experiment (blue line in **A**). Basal OCR was obtained in BSA-supplemented media containing 10 mM glucose+pyruvate. Carboxyatractyloside and oligomycin (CAT+Omy; 12.5 μM and 20 μg/mL) were then added to determine OCR not dependent on ATP production (leak). Maximal respiratory rate was achieved by titration with FCCP (20 μM), following which non-mitochondrial respiration was determined by adding rotenone (Rot, 2.2 μM). From each recording we determined the OCR values presented in **(B)**. Two-Way ANOVA analysis revealed a significant effect of age on both maximal ($F = 4.69$; $P = 0.0368$) and sparing capacity ($F = 7.39$; $^{***}p = 0.01$). In **(C)** one can observe that the drop in [O_2] from the medium bathing the hippocampal slice (aCSF bubbled with 95%O_2/5%CO_2 gas mixture, at 32°C) is significantly decreased in old-aged 3xTg-AD, further supporting respirometry data showing decrease in basal metabolic rate. This drop was determined electrochemically using carbon fiber microelectrodes held at −0, 8 V vs. Ag/AgCl and lowered from the perfusion media into the slice core gradually (see Ledo et al., 2005 for detailed description).

changes in either •NO concentration dynamics or cellular redox environment toward a more oxidative status (which promotes production of reactive oxygen and nitrogen species), not only detours •NO from its physiological role but also precipitates the production of highly oxidative and nitrosative sprecies such as peroxynitrite and dinitrogen trioxide (Heinrich et al., 2013). In line with this hypothesis, increase in tyrosine nitration is observed in the brains of AD patients when compared to healthy age-matched individuals, indicating changes in •NO bioactivity (Fernandez-Vizarra et al., 2004).

Using hippocampal slices to measure both NMDA-evoked •NO production and changes in O_2 profiles in the CA1 subregion, we have previously shown that •NO may act as a modulator of neurometabolic rate upon stimulation of glutamatergic transmission (Ledo et al., 2010). In hippocampal slices obtained from old-age 3xTg-AD mice, and looking specifically at the CA1 pyramidal layer, we observe decreased •NO upon activation of NMDA receptor (unpublished data), which most likely results from changes in •NO bioavailability due to its rapid reaction with species such as superoxide radical to produce peroxynitrite. As a consequence, we also observed that the tight coupling between neuronal-•NO and inhibition of O_2 consumption as shown in healthy subjects is lost, suggesting that •NO is no longer capable or available to act as the master regulator of neurometabolic coupling.

Conclusion

An increasing amount of evidence supports the notion that Alzheimer's disease is a multifaceted pathology that goes far

beyond the amyloid pathology. As a major risk factor for AD, aging shares many common features associated with functional decline, namely neurovascular and neurometabolic alterations. Although the causal role of these changes in AD pathology remains controversial, it seems increasingly certain that they significantly impact the progression of neuronal dysfunction. Furthermore, the imbalance in the regulation of the neurovascular and neurometabolic coupling, resulting from cerebrovascular dysfunction, appear to be precocious events in neurodegeneration and brain aging. This shift in paradigm and the role of vascular redox status of brain microcirculation may be crucial for development of adequate therapeutically strategies that hamper cognition defects and neurodegeneration.

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OPINION
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Uncertainties in pentose-phosphate pathway flux assessment underestimate its contribution to neuronal glucose consumption: relevance for neurodegeneration and aging

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The Impact of PPP in Redox and Energy Conservation

The pentose-phosphate pathway (PPP) promotes the oxidative decarboxylation of glucose-6-phosphate (G6P) in two consecutive steps, catalyzed by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), yielding ribulose-5-phosphate (Ru5P) (**Figure 1**) (Wamelink et al., 2008). These steps constitute the so-called oxidative PPP branch, where the redox energy of G6P is conserved as NADPH(H^+). Together with other NADPH(H^+) regenerating systems, such as NADP-dependent isocitrate dehydrogenase and malic enzyme (ME), the oxidative PPP branch represents the most important source of reducing equivalents for (i) antioxidant enzymes, such as glutathione peroxidases and thioredoxin reductases, and (ii) fatty acid synthase (Dringen et al., 2007). Ru5P is isomerized into ribose-5-phosphate (R5P), which serves either as the precursor for nucleotide biosynthesis, or it continues metabolism through the non-oxidative PPP branch. In the latter, R5P epimerize into xylulose-5-phosphate (Xu5P), with which it transketolases producing sedoheptulose-7-phosphate (S7P) plus glyceraldehyde-3-phosphate (G3P). In turn, S7P and G3P transaldolase to form fructose-6-phosphate (F6P) and erythrose-4-phosphate (E4P). E4P is then transketolated with Xu5P forming F6P and G3P. Thus, through the PPP, three moles of G6P yield three CO_2, two F6P, and one G3P. Since PPP-derived F6P and G3P are glycolytic intermediates too, they can follow conversion into pyruvate. Thus, glycolysis and PPP are two different pathways that share common pools of F6P and G3P intermediates. Accordingly, G6P converted into pyruvate through the PPP conserves both the redox and the energetic values of glucose, highlighting a yet unrecognized high impact of PPP flux activity in redox/energy conservation.

Abbreviations: E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; F6B, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; R5P, ribose-5-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; F26BP, fructose-2,6-bisphosphate; ME, malic enzyme; PEPCK, phosphoenolpyruvate carboxykinase; PFK1, 6-phosphofructo-1-kinase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3; PGI, phosphoglucose isomerase; 6PGD, 6-phosphogluconate dehydrogenase; PPP, pentose-phosphate pathway; Ru5P, ribulose-5-phosphate; TCA, tricarboxylic acid; S7P, sedoheptulose-7-phosphate; Xu5P, xilulose-5-phosphate.

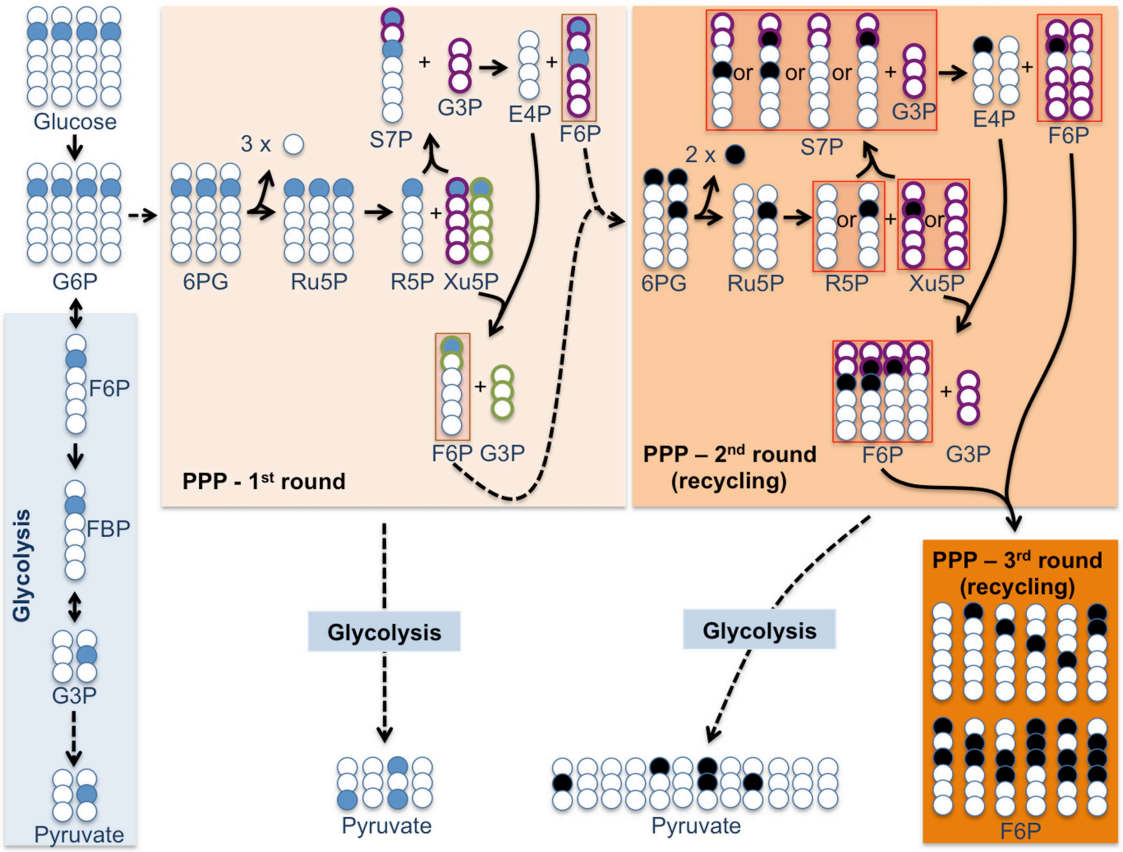

FIGURE 1 | ^{13}C-Pyruvate labeling pattern from [2-^{13}C]glucose by *recycling* PPP underestimates PPP contribution to glucose metabolism. Labeling pattern in ^{13}C-pyruvate originated from glycolysis ([2-^{13}C]pyruvate) differs from that originated from a first round of PPP ([3-^{13}C]- and [1,3-$^{13}C_2$]pyruvate), thus helping to estimate the contribution of both pathways in glucose utilization (blue carbons). However, some cell types, such as neurons, express high PGI activity responsible for high equilibration rate of F6P with G6P, hence leading to a *recycling*-like PPP (PPP – second and third rounds). Identification of these recycling-PPP ^{13}C-labeled intermediates (black carbons) identifies a more complex pattern of ^{13}C-pyruvate labeling ([1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]pyruvate after the second PPP round; pattern after a third PPP round is omitted for simplicity) that overestimates glycolysis, hence underestimating the contribution of PPP to glucose utilization. Please note that [1-^{13}C]pyruvate could also come from TCA cycle (pyruvate *re-recycling*; see text). However, this formation represents as low as one-twelfth of the pyruvate originated from the second round of PPP, thus likely minimally affecting to PPP activity estimation. Therefore, labeling in ^{13}C-Ru5P and ^{13}C-R5P ([2-^{13}C]-, [1,2-$^{13}C_2$]-, [1,2,3-$^{13}C_3$]-, etc.) after the second PPP round can be used for an accurate estimation of PPP in highly recycling cells.

Radioactive PPP Assessment Ex Vivo

Several approaches have been used to estimate the fraction of G6P that is metabolized through the PPP. For experiments performed *ex vivo* (cultured and freshly isolated cells, or tissue slices), radiometric $^{14}CO_2$ detection after incubation of the biological samples in the presence of [1-^{14}C]glucose, under initial velocity conditions, reports flux through the oxidative branch of the PPP. However, since C_1-G6P is also decarboxylated in the tricarboxylic acid (TCA) cycle, the $^{14}CO_2$-value, taken alone, intrinsically over-estimates PPP activity. To overcome this drawback, a parallel incubation under identical conditions needs to be performed to quantify $^{14}CO_2$ collected from [6-^{14}C]glucose, since C_6-G6P is exclusively decarboxylated in the TCA after G6P is converted into pyruvate both through glycolysis and PPP. Thus, the difference in the rate of $^{14}CO_2$ produced from [1-^{14}C]glucose and that from [6-^{14}C]glucose represents the flux of $^{14}CO_2$ exclusively produced at the oxidative PPP branch (Hothersall et al., 1979; Larrabee, 1989). Whilst this approach is suitable for *ex vivo* analyses, it is technically tedious for *in vivo* PPP assessments, as CO_2 collection *in vivo* is hardly quantitative.

^{13}C-NMR PPP Assessment *In Vivo*

Several approaches have been used to assess the proportion of G6P that is metabolized through the PPP *in vivo*—which are also useful for *ex vivo*. A widely used method is essentially based on the analysis of ^{13}C abundance of certain carbon-atoms in lactate upon perfusion (*in vivo*) or incubation (*ex vivo*) with [2-^{13}C]glucose. Thus, the abundance of [3-^{13}C]lactate is assumed to be exclusively originated *via* PPP metabolism, whereas the abundance of [2-^{13}C]lactate, *via* glycolysis (**Figure 1**)

(Brekke et al., 2012). In a slightly different approach based on the use of [1,2-$^{13}C_2$]glucose, the abundances of [3-^{13}C]lactate and [2,3-^{13}C]lactate are considered to be originated *via* PPP and glycolysis, respectively (Jalloh et al., 2015). [1,6-$^{13}C_2$,6,6-$^{2}H_2$]glucose has also been used to measure cerebral PPP activity *in vivo* (Ben-Yoseph et al., 1995) on the bases that it produces [3-^{13}C]lactate and [3-^{13}C,3,3-$^{2}H_2$]lactate through glycolysis, but [3-^{13}C,3,3-$^{2}H_2$]lactate and unlabeled lactate through PPP (Ross et al., 1994). The ratios of these lactate isotopomers can be quantified using gas chromatography/mass spectrometry for calculation of PPP activity, and expressed as the percentage of glucose metabolized to lactate that passed through the PPP (Ross et al., 1994; Ben-Yoseph et al., 1995). These analyses provide information on the relative contribution of a metabolic pathway within overall glucose utilization, in contrast to the initial velocity analyses, which informs on absolute metabolic flux values. Whilst such a difference might account for the controversies in PPP activity values reported by different laboratories (Herrero-Mendez et al., 2009; Brekke et al., 2012; Rodriguez-Rodriguez et al., 2013), in our opinion there are some methodological concerns, as explained below.

Occurrence of Recycling PPP

A critical point that is often overlooked is the occurrence of G6P recycling from PPP-derived F6P. Thus, phosphoglucose isomerase (PGI) is a near-equilibrium enzyme that has been shown to be highly active at converting F6P into G6P in certain cells and/or tissues, such as, e.g., neurons (Gaitonde et al., 1989), which can re-enter the PPP (**Figure 1**). In such *recycling*-like PPP, only the PPP-derived G3P fully escapes from this cycle to be transformed into pyruvate trough glycolysis. High recovery of G6P from PPP-derived F6P may represent a bioenergetics advantage, since the redox energy of glucose can be conserved as NADPH(H^+) at the expense of only one carbon (C_1) per G6P. It should be noted that, in certain cells such as neurons, 6-phosphofructo-1-kinase (PFK1, which converts F6P into fructose-1,6-bisphosphate or F16BP) represents a glycolysis bottleneck (Almeida et al., 2004). Thus, PFK1 *in situ* activity is very low in neurons when compared, e.g., with neighbor astrocytes (Almeida et al., 2004). Such a low PFK1 activity is due to the virtual absence of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) (Herrero-Mendez et al., 2009), responsible for the formation of fructose-2,6-bisphosphate (F26BP)—the most potent positive PFK1 effector. In contrast, astrocytes abundantly express PFKFB3 protein—and F26BP concentration—and, accordingly, a high *in situ* PFK1 activity (Almeida et al., 2004). It is therefore likely that, whereas PPP-derived F6P is preferentially recycled in the PPP in neurons, it is preferably converted into lactate in astrocytes. In fact, the *in situ* PFK1 activity is ∼four-fold lower in neurons when compared with astrocytes (Almeida et al., 2004). Therefore, we hypothesize that, in neurons, (i) most PPP-derived F6P is recycled back into the PPP, and (ii) the vast majority of G3P pool is originated directly from PPP activity. However, in astrocytes, G3P pool is originated from (i) G6P through glycolysis, (ii) PPP-derived F6P then *via* glycolysis, and (iii) directly from PPP activity. On our opinion, these considerations may have implications when interpreting the ^{13}C-labeling data for the estimation of PPP activity, as indicated below.

PPP Assessment is Intrinsically Underestimated

When [2-^{13}C]glucose infusion and/or incubation is used, [1-^{13}C]- and [1,3-$^{13}C_2$]lactate abundances are exclusively PPP-derived, whereas [2-^{13}C]lactate abundance is glycolysis-derived (**Figure 1**). Therefore, the relative abundance of [1-^{13}C]- and [1,3-$^{13}C_2$]lactate when compared with total lactate is often considered to be the proportion of glucose metabolized through the PPP (Brekke et al., 2012). However, [1,3-$^{13}C_2$]F6P, formed in the PPP from [2-^{13}C]glucose, when converted into [1,3-$^{13}C_2$]G6P *via* PGI activity (F6P ⟶ G6P-forming), which is very high in neurons (Gaitonde et al., 1989), can produce [2-^{13}C]F6P in the second PPP round (**Figure 1**). Thus, as from the second PPP round and thereafter, such [2-^{13}C]F6P will also yield [2-^{13}C]lactate, largely over-estimating glycolysis. In addition, the degree of *recycling* PPP activity is not addressed when measuring [3-^{13}C,3,3-$^{2}H_2$]lactate plus unlabeled lactate that is formed from [1,6-$^{13}C_2$,6,6-$^{2}H_2$]glucose (Ben-Yoseph et al., 1995), since this approach does not address the proportion of, and rate at which F6P is recycled back into the PPP. It may therefore be concluded that the methodological approaches to estimate recycling PPP activity may be intrinsically underestimated in neurons. We hypothesize that the degree of recycling PPP activity would be best indicated by the levels of PPP-specific intermediates (**Figure 1**). In view that [1,2-$^{13}C_2$]Ru5P is exclusively formed by recycling PPP from [2-^{13}C]glucose when entering the third round, and [1,2,3-$^{13}C_3$]Ru5P when entering the fourth round, we propose to determine the relative abundance of mono-labeled vs. multiple labeled ^{13}C-R5P +^{13}C-Ru5P, which is feasible by liquid chromatography/mass spectroscopy (LC/MS).

Overestimation of Glycolysis

It is a widely held custom to determine total lactate released from cells as an index of glycolysis. However, given that—at least under several circumstances—the precise proportion of G6P that is metabolized through PPP to lactate is uncertain, total lactate as an index of glycolysis should be taken with caution. We rather propose that lactate release should be considered as an overall index for glucose utilization—i.e., glycolysis plus PPP. To estimate glycolysis, the use of [5-^{3}H]glucose conversion into $^{3}H_2O$, which takes place at enolase, is often used to assess the rate of glycolysis (Neely et al., 1972). However, since enolase cannot distinguish the origin—glycolysis or PPP—of its substrate—2-phosphoglycerate—, the production of $^{3}H_2O$ from [5-^{3}H]glucose does not help to clarify the specific contribution of glycolysis to glucose utilization. Thus, the use of [5-^{3}H]glucose could greatly overestimate the rate of glycolysis in neurons, as it has been shown in the rat heart (Goodwin et al., 2001), which in turn underestimates PPP. We encourage the use of [3-^{3}H]glucose to estimate glycolysis, since ^{3}H of C_3-glucose

interchanges with water at aldolase (Katz et al., 1965), hence reducing the contribution of PPP to the collected ^3H_2O. Since PFK1 *in situ* activity is very low in neurons (Herrero-Mendez et al., 2009), the [3-^{3}H]glucose approach reflects more accurately glycolysis than the [5-^{3}H]glucose one. Using [3-^{3}H]glucose, we have reported that primary cortical neurons produce ^3H_2O at a rate that is ∼four-fold lower than in primary cortical astrocytes (Almeida et al., 2004). However, the proportion of glucose that is metabolized through glycolysis in neurons can be overestimated. It has been widely reported that [1-^{13}C]glucose perfusion in rodents renders, in the brain, a higher ^{13}C-specific enrichment in [4-^{13}C]glutamate (mainly present in neurons) compared to [4-^{13}C]glutamine (mainly present in astrocytes) (Fitzpatrick et al., 1990; Kanamatsu and Tsukada, 1994; Bouzier et al., 1999). When comparing the specific enrichment of [4-^{13}C]glutamate and [1-^{13}C]glucose, it was calculated that ∼90% of the [4-^{13}C]glutamate was originated from the glycolytic metabolism of [1-^{13}C]glucose. The ∼10% loss in specific enrichment of [4-^{13}C]glutamate is usually attributed to a loss of $^{13}C_1$ in the PPP. Unfortunately, this interpretation does not take into account that [3-^{13}C]lactate can be actively used by neurons and converted into [4-^{13}C]glutamate *in vivo* (Bouzier et al., 2000), since glycolytically-generated lactate is shuttled from astrocytes to neurons (Pellerin and Magistretti, 1994; Bouzier-Sore et al., 2006), therefore underestimating *in vivo* neuronal PPP.

Pyruvate Re-Cycling

Pyruvate can be re-generated from the TCA cycle intermediates malate and oxaloacetate through ME and PEPCK, respectively. Such a pyruvate *re-cycling* has been shown to occur in neurons (Cerdan et al., 1990; Cruz et al., 1998), where malic enzyme is expressed profusely (Vogel et al., 1998; McKenna et al., 2000), although its occurrence in astrocytes has also been proposed (Olstad et al., 2007). Therefore, when using [2-^{13}C]glucose to estimate the PPP activity, [2-^{13}C]pyruvate entering the TCA cycle will yield [1-^{13}C]pyruvate from *re-cycling*, which will be undistinguishable from that returning from the second PPP round (**Figure 1**). This further reinforces our proposal that PPP should be estimated by measuring ^{13}C incorporation into the five carbon-atom sugars, instead of that into lactate or pyruvate.

PPP is an Advantage for Neurons that May Failure in Neurodegeneration and Aging

Neurons are deficient in antioxidant glutathione (Bolaños et al., 1995), hence recycling of reduced glutathione (GSH) from its oxidized form (GSSG) is critical for their survival. Since NADPH(H^+) is essential for this process, the re-cycling version of the PPP—which recovers a considerable proportion of G6P—explains both the low glucose consumption and the efficient GSH regenerating activity of neurons. In contrast, astrocytes express higher PPP-rate limiting step G6PD, and PPP activity, than neurons (Garcia-Nogales et al., 2003; Herrero-Mendez et al., 2009), besides higher glycolysis (Almeida et al., 2004; Herrero-Mendez et al., 2009). This is likely indicating that both non-recycling PPP plus glycolysis contribute to glucose consumption in astrocytes. Accordingly, G6P recovery through re-cycling PPP in neurons represents an advantage for neuronal survival. A large body of evidence is now showing, by PET studies, decreased glucose consumption—actually reflecting glucose uptake—in the human brain during aging and neurodegeneration (Chen and Zhong, 2013). This observation has been widely interpreted as a putative cause of limited energy production in neurons. However, before we uncover the actual contribution of PPP to glucose consumption by the brain cells, particularly neurons and astrocytes, such an assertion remains uncertain. We herein opine that the decrease in glucose utilization occurring in aging and neurodegeneration, far from merely causing a bioenergetics problem, would be responsible for an oxidative damage due to failed PPP-derived NADPH(H^+) regeneration in neurons. Furthermore, we also propose that the impaired glucose utilization observed in PET studies in neurodegeneration and aging reflects, mainly, reduced glucose consumption in astrocytes. This will, in turn, result in reduced lactate released for neuronal conversion into either energy or neurotransmitter glutamate. Either hypothesis, i.e., low neuronal PPP activity causing failed antioxidant capacity, low lactate supply by astrocytes, or a combination of both, may have been largely overlooked as the cause of both bioenergetics and antioxidant neuronal damage during aging and neurodegeneration. More in-depth studies to overcome the methodological drawbacks herein highlighted should be undertaken in order to unveil the actual contribution of PPP to neuronal survival *in vivo*.

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REVIEW
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Triad of Risk for Late Onset Alzheimer's: Mitochondrial Haplotype, APOE Genotype and Chromosomal Sex

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Brain is the most energetically demanding organ of the body, and is thus vulnerable to even modest decline in ATP generation. Multiple neurodegenerative diseases are associated with decline in mitochondrial function, e.g., Alzheimer's, Parkinson's, multiple sclerosis and multiple neuropathies. Genetic variances in the mitochondrial genome can modify bioenergetic and respiratory phenotypes, at both the cellular and system biology levels. Mitochondrial haplotype can be a key driver of mitochondrial efficiency. Herein, we focus on the association between mitochondrial haplotype and risk of late onset Alzheimer's disease (LOAD). Evidence for the association of mitochondrial genetic variances/haplotypes and the risk of developing LOAD are explored and discussed. Further, we provide a conceptual framework that suggests an interaction between mitochondrial haplotypes and two demonstrated risk factors for Alzheimer's disease (AD), apolipoprotein E (APOE) genotype and chromosomal sex. We posit herein that mitochondrial haplotype, and hence respiratory capacity, plays a key role in determining risk of LOAD and other age-associated neurodegenerative diseases. Further, therapeutic design and targeting that involve mitochondrial haplotype would advance precision medicine for AD and other age related neurodegenerative diseases.

Keywords: mitochondria, haplogroup, Alzheimer's disease, APOE, sex

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INTRODUCTION

A central role of mitochondria in age-related metabolic and neurodegenerative diseases has long been proposed, and an association between mitochondrial dysfunction and Alzheimer's disease has been long proposed across multiple investigative strategies from analysis of human tissue to cell model systems (Beal, 1996; Gibson et al., 2000; Trimmer et al., 2000; Swerdlow and Khan, 2004; Bubber et al., 2005; Wallace, 2005; Lin and Beal, 2006; Brinton, 2008, 2009; Khusnutdinova et al., 2008; Simpkins et al., 2008; Coskun et al., 2012; Brinton et al., 2015). The "mitochondrial cascade hypothesis", originally proposed by Swerdlow and Khan (2004) as an explanation for late onset Alzheimer's disease (LOAD), proposed that inherited mitochondrial genetic variations and mutations accumulated from aging should occupy the apex of the disease cascade. Briefly, this hypothesis maintained that mitochondrial genetic variations and mutations lead to deficient electron transport chain function, resulting in less ATP production, increased free radical production, disrupted

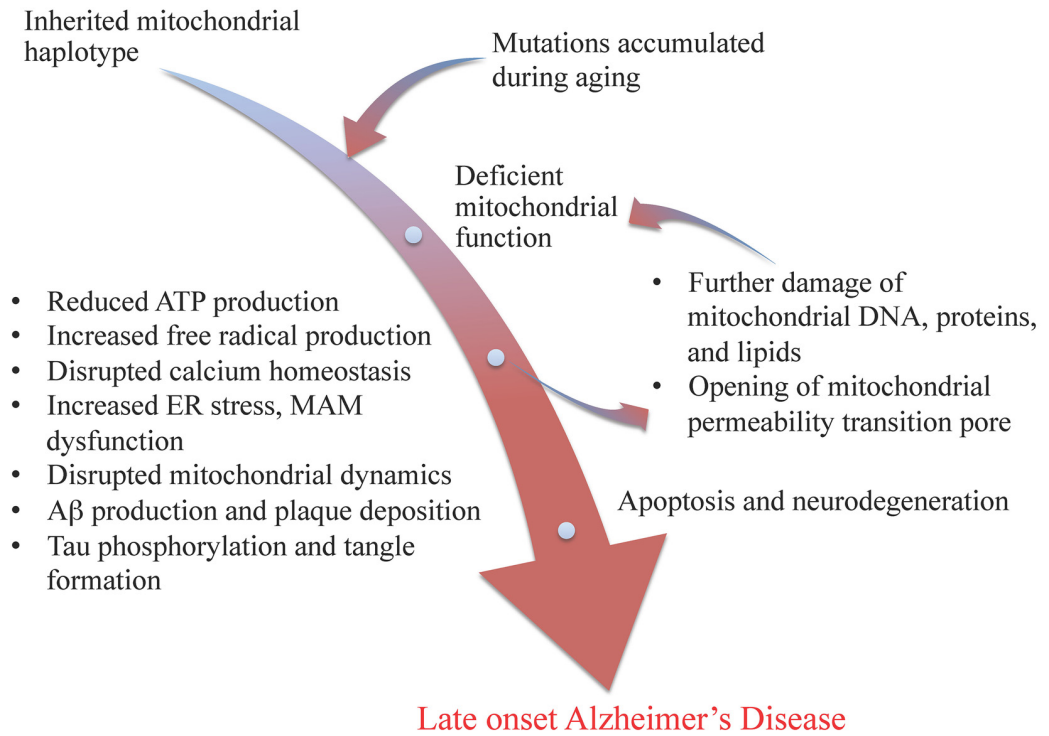

FIGURE 1 | Modified mitochondrial cascade of late-onset Alzheimer's disease (LOAD). Inherited mitochondrial genetic variations and accumulated mutations during aging lead to deficiency in mitochondrial functions, initiating a cascade of events including reduced ATP production, increased free radical formation and ER stress, disrupted mitochondrial associated membrane (MAM) function and mitochondrial dynamics, as well as amyloid beta plaque and tau tangle formation, which result in further damages of mitochondria and ultimately leads to apoptosis and neurodegeneration, such as LOAD.

calcium homeostasis, beta amyloid production and plaque deposition, and tau phosphorylation and tangle formation. These results in turn lead to further damage of mitochondrial DNA, proteins, and lipids, and the opening of mitochondrial permeability transition pore, which ultimately leads to cell death and neurodegeneration (see **Figure 1**). Recent studies also indicated disrupted crosstalk between mitochondria and endoplasmic reticulum (ER) via mitochondrial associated membrane (MAM), as well as abnormal mitochondrial dynamics in the etiology of Alzheimer's disease (AD; Trimmer et al., 2000; Baloyannis et al., 2004; Paillusson et al., 2013; Burté et al., 2015; Zhang et al., 2016; see **Figure 1**).

Here we review the link between mitochondrial dysfunction and the risk of AD, the contribution of mitochondrial genetic variances to the risk, and how the risk is modulated by other factors, such as apolipoprotein E (APOE) genotype and sex differences.

MITOCHONDRIAL GENOME AND HAPLOTYPES

Unlike many other organelles, mitochondria have their own genome. The human mitochondrial genome is a circular set of 16,569 base pairs encoding 37 genes. Thirteen of these genes encode protein subunits required for four of the five electron transport chain complexes: complex I (NADH ubiquinone oxidoreductase), complex III (cytochrome bc1 complex), complex IV (cytochrome c oxidase), and complex V (ATP synthase); two encode rRNAs for mitochondrial ribosomes (12S and 16S), and 22 encode tRNAs (see **Figure 2**).

Mitochondrial retention of their own genome throughout evolution solves two cell biology problems. First, the 13 electron transport subunits coded by mitochondrial DNA solves the problem that if they were generated by nuclear DNA, they would not cross the inner mitochondria membrane due to their high hydrophobicity (Popot and de Vitry, 1990). Second, the eukaryotic mitochondrial genome is transcribed and translated quite differently than the nuclear genome (Mercer et al., 2011). The genetic system of the mitochondria is transcribed as precursor polycistronic transcripts that are subsequently cleaved to generate mRNAs, tRNAs and rRNAs (Mercer et al., 2011). The mRNAs devoted to generating the 13 catalytic subunits required for oxidative phosphorylation are further translated using several non-universal codons unique to the mitochondrial translation machinery (Watanabe, 2010; Watanabe and Yokobori, 2011).

Like nuclear DNA, mitochondrial DNA undergoes mutation, though at a much higher rate (Miyata et al., 1982; Wallace et al., 1987), likely due to higher replication rate, a more

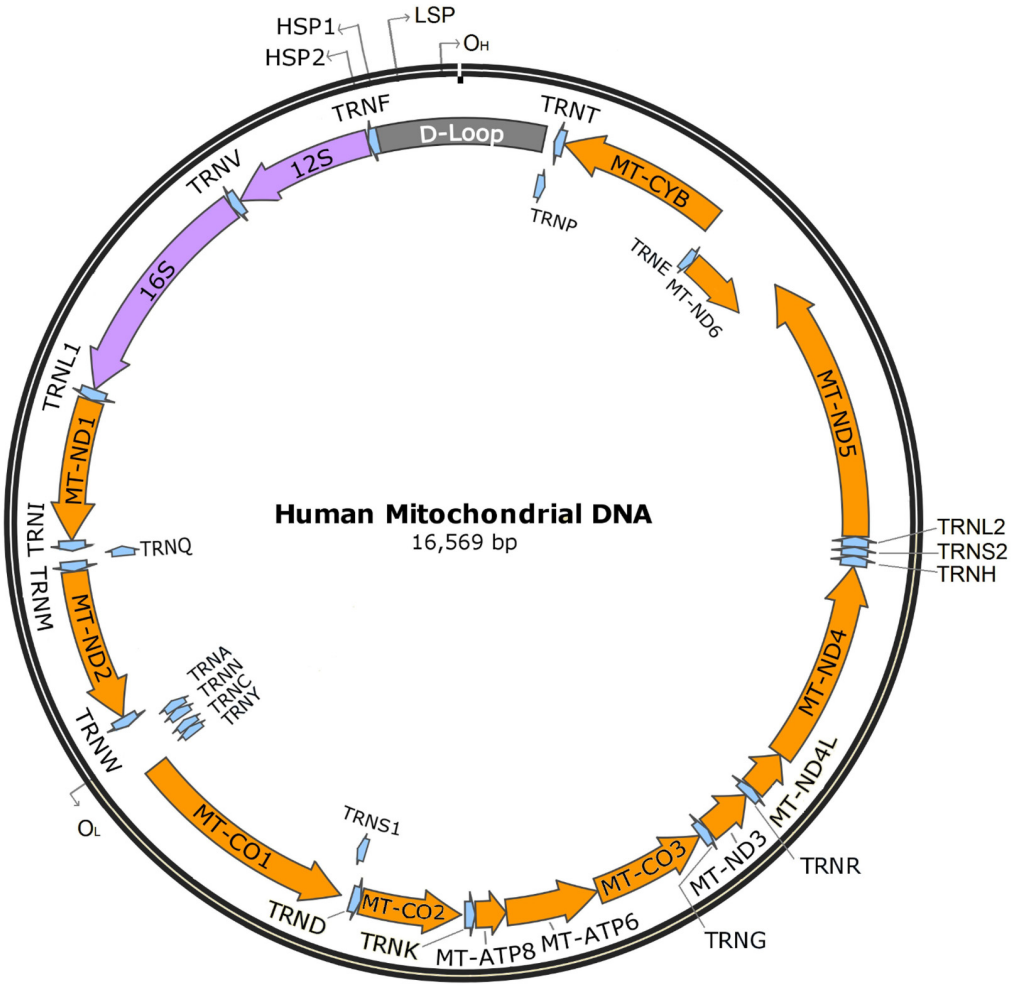

FIGURE 2 | Human mitochondrial DNA. Orange indicates protein-encoding genes (13), purple indicates mitochondrial rRNAs (2), blue indicates tRNAs (22), and gray indicates the D-loop. O_H: heavy strand origin, O_L: light strand origin, HSP1: major heavy strand promoter, HSP2: minor heavy strand promoter, LSP, light strand promoter.

mutagenic environment, and less efficient DNA repair (Xu et al., 2012). Unlike the nucleus, the mitochondrial DNA repair mechanism is largely limited to base excision repair. Mismatch as a result of either recombination or repair can lead to single nucleotide polymorphisms (SNPs).

Clusters of specific SNPs in the mitochondrial genome define mitochondrial haplogroups that reflect maternal lineage (Giles et al., 1980; Torroni et al., 1992). For example, the four lineages specific for sub-Saharan Africa are L0, L1, L2 and L3, and haplogroups A, B, C, D, G and F are common in Asia (Stewart and Chinnery, 2015). The major haplogroups within descendants of European ancestry are haplogroups H, I, J, K, M, T, U, V, W and X (Torroni et al., 1996). These haplogroups can be further classified into subhaplogroups or clustered together into superhaplogroups, such as superhaplogroup HV, JT, UK.

MITOCHONDRIAL DYSFUNCTION AND INCREASED RISK OF ALZHEIMER'S DISEASE

AD is a systemic disease with multiple etiologies (Morris et al., 2014). Early evidence linking mitochondrial dysfunction to AD dates back to a 1987 study by Sims et al. (1987), who found a reduced rate of oxygen uptake in the presence and absence of ADP in frontal neocortex of postmortem confirmed AD cases, indicating potential mitochondrial uncoupling in AD patients (Sims et al., 1987).

More direct evidence for the association between mitochondrial dysfunction and AD came later from studies on the activity of electron transport chain enzymes in AD patients and post mortem AD brain tissues. In the early 1990's, reduced cytochrome c oxidase (complex IV) activity was observed in both platelets and postmortem AD brain

(Parker et al., 1990, 1994). The reduction of cytochrome c activity in AD brain was later refined to the temporal cortex and hippocampus (Maurer et al., 2000). Supporting these findings, reduced mRNA levels of cytochrome c oxidase subunit 1 and 3 were observed in AD mid temporal gyrus and cytochrome c oxidase subunit 2 in AD hippocampus (Chandrasekaran et al., 1994; Aksenov et al., 1999). Furthermore, in both AD temporal and parietal cortices, protein levels of cytochrome c oxidase subunits were reduced, especially those encoded by mitochondria DNA (Kish et al., 1999). Although no reduction of cytochrome c oxidase content was observed in platelets of AD patients, cytoplasmic hybrids (cybrids) containing exogenous mitochondria extracted from platelets of AD patients showed less cytochrome c oxidase activity compared to cybrids harboring mitochondria from age-matched controls (Davis et al., 1997; Sheehan et al., 1997; Cardoso et al., 2004). Alternations in gene expression, protein level, and activity of other electron transport chain complexes have also been reported in AD tissues, though the evidence was less compelling and sometimes contradicted (Schagger and Ohm, 1995; Chandrasekaran et al., 1997; Aksenov et al., 1999; Kim et al., 2000; Bosetti et al., 2002; Bubber et al., 2005; Valla et al., 2006).

Upstream to electron transport chain, impairment of the tricarboxylic acid (TCA) cycle enzymes was observed in AD brain. Autopsy-confirmed AD brain had significantly reduced activity in pyruvate dehydrogenase complex, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase complex, whereas the activity of succinate dehydrogenase and malate dehydrogenase were increased (Bubber et al., 2005). The severity of enzyme activity impairment was also correlated with the clinical severity of Alzheimer's pathology (Bubber et al., 2005). Decreased mitochondrial respiratory capacity was consistent with the higher level of lactate and lower key substrates for TCA cycle in cerebrospinal fluid and blood in AD patients (Redjems-Bennani et al., 1998; Mancuso et al., 2003).

Consistent with deficits in mitochondrial respiratory capacity, AD brain had elevated levels of peroxidation products in the frontal cortex, as well as decreased levels of superoxide dismutase, a radical defensive enzyme, in the frontal cortex, hippocampus, and cerebellum (Richardson, 1993). Increased oxidative stress is consistent with free radical damage of mitochondrial components and loss of mitochondrial membrane potential as observed in cybrids harboring mitochondria with AD origin (Cassarino et al., 1998; Trimmer et al., 2000).

Besides altered bioenergetic capacity, the crosstalk between mitochondria and the ER via MAM, which regulates many key functions of mitochondria, such as calcium uptake, phospholipid exchange, intracellular trafficking, ER stress and mitochondrial biogenesis, was also disrupted in AD (Paillusson et al., 2013; Burté et al., 2015).

Beyond changes in mitochondrial function, distribution and morphology of mitochondria were also different in AD patients. Neurons from AD brain harbored mitochondria of smaller sizes and disrupted mitochondrial cristae morphology, and cybrids created using mitochondria from sporadic AD patients also showed swollen mitochondria and less cristae (Trimmer et al., 2000; Baloyannis et al., 2004). The fission and fusion cycle of mitochondria also seemed disrupted in the hippocampus of AD brain (Zhang et al., 2016). The number of mitochondria was also significantly reduced, likely as a result of increased mitochondria degradation, turnover and autophagy (Hirai et al., 2001).

AD has a pattern of maternal inheritance, where inheritance of AD from mothers are more frequent than from fathers (Duara et al., 1993). The observation of maternal pattern of Alzheimer's inheritance has been replicated over many decades by multiple groups, and has been linked to deficits in mitochondrial respiration and glucose metabolism apparently early in the aging process (Edland et al., 1996; Mosconi et al., 2011; Liu et al., 2013). In cognitively normal individuals, those with a maternal history of LOAD showed decline in platelet cytochrome c oxidase activity, compared to those with a paternal or no family history of the disease (Edland et al., 1996; Mosconi et al., 2011). In this same cohort, Mosconi et al. (2011) observed significantly lower brain glucose metabolism in persons with a maternal history of Alzheimer's relative to those with a paternal or no family history of the disease. These data coupled with maternal inheritance of the mitochondrial genome strongly support a role for mitochondrial genetic variances in the etiology of the disease.

MITOCHONDRIAL HAPLOTYPE IS ASSOCIATED WITH DIFFERENT RESPIRATORY, METABOLIC AND BIOENERGETIC PHENOTYPES

Mitochondrial genetic background can also affect metabolism and bioenergetic function. Resting metabolic rate (RMR) and total energy expenditure (TEE) were measured in 294 participants in the health, aging and body composition study (Health ABC; Tranah et al., 2011), including participants of cluster L, which contains common African haplogroups, and cluster N, which contains common European haplogroups (Tranah et al., 2011). Compared to N, cluster L had significantly lower RMR and TEE. Specifically, haplogroups L0, L2 and L3 had significantly lower RMR than haplogroup H and superhaplogroups UK and JT; haplogroup L3 had significantly lower TEE than haplogroup H and superhaplogroups UK and JT; haplogroup L2 had significantly lower TEE than haplogroup H and superhaplogroup JT (Tranah et al., 2011; see **Table 1**). In a cohort of healthy Spanish males, haplogroup J participants had significantly lower maximum oxygen consumption (VO_{2max}) than non-J participants (Marcuello et al., 2009). This difference was later confirmed in an independent cohort of healthy Spanish males, where haplogroup H was determined to be the driving force for the difference (Martínez-Redondo et al., 2010; see **Table 1**).

The underlying cellular mechanism of the observed differences across different mitochondrial haplogroups was

TABLE 1 | Mitochondrial haplogroups/superhaplogroups differentially associated with respiratory phenotypes.

	Relatively high	Relatively low	Reference
RMR	H, UK, JT	L2, L3, L3	Tranah et al. (2011)
TEE	H, UK, JT	L0, L2	Tranah et al. (2011)
VO_{2max}	H	J	Marcuello et al. (2009) and Martínez-Redondo et al. (2010)

Individuals of haplogroup/superhaplogroups H, UK and JT had higher rest metabolism rate (RMR), total energy differences (TEE) and/or VO_{2max} compared to individuals of cluster L and haplogroup J.

primarily elucidated using trans-mitochondrial cytoplasmic hybrids, or cybrids, which controlled for the nuclear genetic background to reveal mitochondrial variances. An early cybrids study using cultured A539 human lung carcinoma cells harboring either mitochondria of haplogroup H or T failed to identify any differences in bioenergetics function (Amo et al., 2008). However, differences in bioenergetics and mitochondrial function were identified in multiple later studies using different cell lines and mitochondrial haplogroups. Cybrids constructed from osteosarcoma 143B rho0 cells and platelets from healthy Spanish donors of either haplogroup H or superhaplogroup UK were investigated for mtDNA content (Gómez-Durán et al., 2010). Cybrids harboring UK superhaplogroup were found to have lower mtDNA content, lower mt-rRNA level, reduced protein synthesis, and decreased cytochrome c oxidase amount (Gómez-Durán et al., 2010). UK cybrids also had lower mitochondrial inner membrane potential and higher mitochondrial uncoupling, indicating potentially lower respiratory capacity and reduced ATP production (Gómez-Durán et al., 2010). Similar results were obtained in a later study in middle-age Caucasian males, where OXPHOS capacity normalized to citrate synthase content was found to be reduced by 24% in subjects with haplogroup U background comparing to those with haplogroup H background (Larsen et al., 2014). In 2013, cybrids constructed from human retinal epithelial cell line ARPE-19 and either haplogroup H or J mitochondria showed reduced ATP production and glycolysis in J cybrids (Kenney et al., 2013). In accordance with the observed reduction in mitochondrial respiration, J cybrids also showed lower ROS production (Kenney et al., 2013). Haplogroup J cybrids with chondrocyte nuclear genetic background also demonstrated lower NO levels than non-J cybrids (Fernández-Moreno et al., 2011). Similarly, major Asian mitochondrial haplogroups are also differentially associated with bioenergetic function (Lin et al., 2012). These associations identified in the human are also evident in animal models ranging from *Drosophila* to mice such that different mitochondrial genetic background is associated with differences in respiratory and metabolic phenotypes, electron transport chain enzyme activities and mitochondrial functions (Pichaud et al., 2012; Scheffler et al., 2012; Latorre-Pellicer et al., 2016).

Although different nuclear genetic background employed in the above studies make it difficult to compare the results across studies, this growing body of evidence supports mitochondrial genetic variance/haplotype as a key factor in the observed differences in bioenergetics and metabolism.

MITOCHONDRIAL GENETIC VARIANCES/HAPLOTYPES AND HAPLOGROUPS AND THE RISK OF ALZHEIMER'S DISEASE

In an early effort to assess the contribution of mitochondrial DNA variances to pathologies of neurodegenerative diseases, researchers identified a non-synonymous SNP in $tRNA^{Gln}$, mt4336C, that had an increased frequency in a Caucasian cohort of late onset Alzheimer's and Parkinson disease patients (Shoffner et al., 1993). The contribution of mt4336C was later confirmed in a different North American Caucasian cohort of AD patients (Hutchin and Cortopassi, 1995). Individuals harboring the mt4336C SNP also tended to harbor the mt16304C SNP, and had a more closely related D-loop sequence, which could be traced back to a single phylogenetic node (Shoffner et al., 1993; Hutchin and Cortopassi, 1995). However, two other studies did not confirm either mt4336C or mt16304C as a risk factor for developing AD in similar populations, when blood samples and leukocytes from clinically diagnosed patients were used instead of histopathologically confirmed postmortem AD brain tissues (Wragg et al., 1995; Zsurka et al., 1998). Today, mt16304C is known as a defining SNP for subhaplogroup H5, and mt4336C a defining SNP for subhaplogroup H5a. The above studies constituted the earliest debate over whether mitochondrial genetic variances can modify the risk of developing AD.

In fact, haplogroup H and superhaplogroup HV, which contains haplogroup H and its subhaplogroups, have been the most reported haplogroup in association with increased risk of developing AD (Chinnery et al., 2000; Edland et al., 2002; van der Walt et al., 2004, 2005; Elson et al., 2006; Fesahat et al., 2007; Mancuso et al., 2007; Maruszak et al., 2009, 2011; Santoro et al., 2010; Coto et al., 2011; Ridge et al., 2012; Fachal et al., 2015; see **Table 2**). A study based on 30 Iranian late onset Alzheimer's patients and 100 controls found that haplogroup H was significantly more abundant in the disease group (Fesahat et al., 2007; see **Table 2**). In a Spanish-Caucasian group, haplogroup H and its defining SNP mt7028C were enriched in LOAD patients compared to controls (Coto et al., 2011; see **Table 2**). In a large Caucasian cohort containing 422 late-onset AD patients and 318 neurologically healthy controls, researchers found that superhaplogroup HV, which contains haplogroup H, had significantly higher presence in LOAD than in control (Maruszak et al., 2011; see **Table 2**). Finally, a meta-analysis pooling data from five previous studies (some studies including early-onset AD patients) also confirmed the association between haplogroup H and superhaplogroup HV and the risk of developing AD (Maruszak et al., 2011; see **Table 2**).

When mitochondrial DNA was sequenced in greater depth, subhaplogroup H5 was significantly associated with AD compared to haplogroup H of central-northern Italians, (Santoro

TABLE 2 | Observed effects of mitochondrial superhaplogroup HV and haplogroup H on risk of Alzheimer's disease (AD).

Haplogroups	Observations	Reference
HV	Increased risk, especially in females	Maruszak et al. (2011)
	No effect	Elson et al. (2006), Maruszak et al. (2009) and Fachal et al. (2015)
H	Increased risk	Fesahat et al. (2007), Maruszak et al. (2009, 2011) and Coto et al. (2011)
	Defining SNP mt7028C increased risk	Coto et al. (2011)
	Defining SNP mt7028C increased risk in females only	van der Walt et al. (2004)
	H5 increased risk, especially in females	Santoro et al. (2010)
	H5 and APOE4 synergistically increased risk	Maruszak et al. (2011)
	H5a defining SNP mt4336C increased risk in APOE4 carriers	Edland et al. (2002)
	H6a1a and H6a1b decreased risk	Ridge et al. (2012)
	No effect	Chinnery et al. (2000), van der Walt et al. (2005), Mancuso et al. (2007) and Fachal et al. (2015)

Effects of haplogroup H defining single nucleotide polymorphisms (SNPs) and its subhaplogroups are listed under haplogroup H.

et al., 2010; see **Table 2**). However, from the Cache county study on aging and memory in Utah residents, subhaplogroups H6a1a and H6a1b were found to be protective against AD (Ridge et al., 2012; see **Table 2**). While the protective role of subhaplogroups H6a1a and H6a1b seems contradictory to the overall risk of haplogroup H, the data predict that the observed risks within haplogroup H may be driven by its subhaplogroups with H5 increases risk of LOAD whereas H6 reduces risk.

The second most studied superhaplogroup is UK, and its member haplogroups U and K, including their subhaplogroups (While haplogroup K is currently recognized as a branch of haplogroup U, early studies classified haplogroups U and K as two parallel haplogroups under superhaplogroup UK. For consistency of referring to previous studies, we will use the earlier classification system throughout this review article; see **Table 3**). In a Utah based ADNI cohort, superhaplogroup UK was identified as a risk factor for AD (Lakatos et al., 2010; see **Table 3**). These findings are in contrast to an earlier study conducted in a Poland-based Caucasian population, where no effect of superhaplogroup UK was observed (Maruszak et al., 2009; see **Table 3**). The disparity may be explained by differences in the distribution of specific subhaplogroups or SNPs in the studied populations. Specifically, while each of the three defining SNPs for haplogroup U (mt11467G, mt12308G and mt12372A) has been identified as a risk factor for AD (Lakatos et al., 2010), subhaplogroup U5a1 and SNP mt16224C, a haplogroup K defining SNP, were shown to be protective (Maruszak et al., 2011; see **Table 3**).

Another common European haplogroup studied for its association with AD is haplogroup T, where one study in French-Canadians found that the frequency of SNPs mt709A and mt15928A, both defining SNPs for haplogroup T, were three times higher in controls than in AD patients, suggesting a protective role of haplogroup T (Chagnon et al., 1999; see **Table 4**). However, the Health, Aging, and Body Composition (Health ABC) study found that haplogroup T had increased risk for dementia when compared to haplogroup H (Tranah et al., 2012). The Health ABC study also found that haplogroup J, also under superhaplogroup JT, was associated with significant decline in cognitive function compared to haplogroup H (Tranah et al., 2012).

In addition to major European haplogroups, several African and Asian haplogroups have also been reported to be associated with the AD or risk of dementia (see **Table 5**). For example,

TABLE 3 | Observed effects of mitochondrial superhaplogroup KU and haplogroups K and U on risk of AD.

Haplogroups	Observations	Reference
UK	Increased risk	Lakatos et al. (2010)
	Decreased risk in males	Maruszak et al. (2011)
	No effect	Maruszak et al. (2009) and Fachal et al. (2015)
K	Defining SNP mt16224C decreased risk	Maruszak et al. (2011)
	Decreased risk in APOE4 carriers	Carrieri et al. (2001) and Maruszak et al. (2011)
	No effect	Chinnery et al. (2000), van der Walt et al. (2005), Elson et al. (2006), Fesahat et al. (2007), Mancuso et al. (2007) and Maruszak et al. (2009)
U	Increased risk	Fesahat et al. (2007)
	Increased risk in males	van der Walt et al. (2004)
	Defining SNPs mt11467G, mt12308G, and mt12372A individually increased risk	Lakatos et al. (2010)
	Decreased risk in females	van der Walt et al. (2004)
	Decreased risk in APOE4 carriers	Carrieri et al. (2001)
	U5a1 decreases risk	Maruszak et al. (2011)
	No effect	Chinnery et al. (2000), van der Walt et al. (2005), Elson et al. (2006), Mancuso et al. (2007), Maruszak et al. (2009) and Fachal et al. (2015)

TABLE 4 | Observed effects of mitochondrial superhaplogroup JT and haplogroups J and T on risk of AD.

Haplogroups	Observations	Reference
JT	Decreased risk in females	Maruszak et al. (2011)
	No effect	Maruszak et al. (2009) and Fachal et al. (2015)
J	Decline in cognitive function comparing to H	Tranah et al. (2012)
	J2b defining SNP mt7476T, mt5633T, and mt15812A increased risk	Chagnon et al. (1999)
	No effect	Chinnery et al. (2000), van der Walt et al. (2005), Elson et al. (2006), Fesahat et al. (2007), Mancuso et al. (2007), Maruszak et al. (2009) and Fachal et al. (2015)
T	Increased risk for dementia comparing to H	Tranah et al. (2012)
	T defining SNP mt709A and mt15928A decreased risk	Chagnon et al. (1999)
	Decreased risk in females	Maruszak et al. (2011)
	No effect	Chinnery et al. (2000), van der Walt et al. (2005), Elson et al. (2006), Fesahat et al. (2007), Mancuso et al. (2007), Maruszak et al. (2009) and Fachal et al. (2015)

in an African American population, haplogroup L1 was found to have increased risk for developing dementia (Tranah et al., 2014). In Asians, subhaplogroups G2a, B4c1 and N9b1 were reported to be associated with AD in Japanese populations, and haplogroup B5 was reported to be associated with AD in Han Chinese (Takasaki, 2008, 2009; Bi et al., 2015; see **Table 5**).

As reviewed here, the field has not reached consensus on the effect of mitochondrial haplogroups on late onset AD (LOAD). Different sets of haplogroups are identified in different studies and different studies could identify opposite effects of the same mitochondrial haplotype on risk of LOAD. Moreover, multiple studies involving Caucasians of European descendant (European descents in UK and US, Tuscans, Spanish and Old order Amish from Indiana and Ohio) did not detect associations between any haplogroup and AD (Chinnery et al., 2000; van der Walt et al., 2005; Elson et al., 2006; Mancuso et al., 2007; Fachal et al., 2015; see **Tables 2–5**). While the controversy may be driven by differences in the distribution of subhaplogroups within the studied population or different DNA sources (e.g., postmortem brain tissue vs. peripheral blood), the discrepancies between studies also suggested that as intriguing as the results are, mitochondrial genetic variances are unlikely to be the sole driving force of LOAD. Further, the most consistent risk factor for LOAD is APOE genotype, but it alone is not an absolute determinant. Below, we determine the relationship between mitochondrial haplogroup and APOE genotype on risk of AD.

TABLE 5 | Association between some Asian mitochondrial haplogroups and the risk of Alzheimer's disease.

Haplogroups	Observations	References
L1	Increased risk	Tranah et al. (2014)
G2a	Increased risk	Takasaki (2008, 2009)
B4c1	Increased risk	Takasaki (2009)
N9b1	Increased risk	Takasaki (2009)
B5	Increases risk	Bi et al. (2015)

Studies listed in chronological order.

THE INTERACTION BETWEEN MITOCHONDRIAL HAPLOGROUP AND APOE GENOTYPE ON RISK OF AD

APOEε4 genotype is a widely recognized risk factor for AD, and has been repeatedly confirmed in the studies reviewed herein (Corder et al., 1993; Poirier et al., 1993; Rebeck et al., 1993; Saunders et al., 1993; Carrieri et al., 2001; Edland et al., 2002; Coto et al., 2011; Maruszak et al., 2011). Further, APOEε4 has been associated with mitochondrial dysfunction and glucose hypometabolism in brain (Reiman et al., 2001, 2004, 2005; Mosconi et al., 2004a,b,c, 2005, 2008; Valla et al., 2010; Wolf et al., 2013). Compared to non-carriers, APOEε4 carriers showed reduced cerebral parietal glucose metabolism among cognitive normal elderlies with family history of AD (Small et al., 1995, 2000). In APOEε4 positive MCI patients, reduced regional cerebral metabolic rate of glucose consumption (rCMRglc) was detected in temporoparietal and posterior cingulate cortex (Mosconi et al., 2004b). In AD patients, more severe hypometabolism was detected in the parietal, temporal, and cingulate areas in APOEε4 carriers than non-carriers (Mosconi et al., 2004c; Drzezga et al., 2005). Brain glucose hypometabolism was also more widespread in APOEε4 positive AD patients (Mosconi et al., 2004a). On the therapeutic side, mild-to-moderate AD patients who are APOEε4 carriers were shown to be less responsive to rosiglitazone, which can improve mitochondrial efficiency and glucose metabolism (Risner et al., 2006; Roses et al., 2007).

In longitudinal studies, APOEε4 carriers had significantly greater rCMRglc decline in the vicinity of temporal, posterior cingulate, and prefrontal cortex, basal forebrain, parahippocampal gyrus, and thalamus (Reiman et al., 2001; Mosconi et al., 2008). Decrease of glucose metabolism was also evident in young and middle-aged APOEε4 carriers in posterior cingulate, parietal, temporal, and prefrontal cortex, as well as thalamus (Reiman et al., 2004; Mosconi et al., 2008). The effect of APOEε4 allele has also been shown to be gene dose dependent with APOEε4

TABLE 6 | Three modes of interactions between APOEε4 status and mitochondrial haplogroups in modulating the risk of AD.

Interactions	Haplogroups/SNPs	Reference
Neutralizing	K	Carrieri et al. (2001) and Maruszak et al. (2011)
	U	Carrieri et al. (2001)
Enabling	mt4336C (H)	Edland et al. (2002)
Synergistic	H5	Maruszak et al. (2011)
	mt7028C (H)	Coto et al. (2011)

Haplogroups K and U could neutralize the risk of APOEε4 on AD. Haplogroup H defining SNP mt4336C was associated with LOAD in APOEε4 carriers only. Subhaplogroup H5 and haplogroup H defining SNP mt7028C could act synergistically with APOEε4 to increase the risk of LOAD.

homozygote carriers showing greater hypometabolic deficit relative to APOEε3/4 heterozygote carriers (Reiman et al., 2005).

At the cellular level, APOEε4 gene expression in human was associated with down-regulation of genes involved in mitochondrial oxidative phosphorylation and energy metabolism (Xu et al., 2006, 2007). APOEε4 gene expression was also found to be associated with lower mitochondrial cytochrome oxidase activity in posterior cingulate cortex among young adults with family history of AD (Valla et al., 2010). Neurons from humanized APOEε4 knock-in mice had significantly lower amount of all five electron transport chain complexes comparing to those from APOEε3 knock-in mice (Chen et al., 2011). Proteomic analysis revealed decreased expression of proteins involved in the TCA cycle, glucose, lipid and amino acid metabolism in APOEε4 knock-in mice (Shi et al., 2014). Further, cytochrome c levels were significantly lower in ApoEε4 mice compared with ApoEε3 mice (Shi et al., 2014). *In vitro* studies also suggested that truncated APOEε4 fragment can interact directly with mitochondria and cause mitochondrial dysfunction and neurotoxicity (Chang et al., 2005; Mahley et al., 2007). Given the association between decreased bioenergetic capacity in brain and the risk of AD, an interaction between APOE genotype and mitochondrial haplotypes is possible.

After stratifying by APOEε4 status, three interesting modes of interactions between APOEε4 status and mitochondrial haplogroups in modulating the risk of AD were apparent (see **Table 6**).

The first mode is a neutralizing effect of mitochondrial haplogroup on the effect of APOEε4 on risk of AD (see **Table 6**). Early studies identified a non-random association between mitochondrial haplogroup and APOEε4 status in AD patients (Carrieri et al., 2001). Specifically, while APOEε4 carriers had significantly higher odds ratio for AD, those belonging to haplogroups K and U did not, indicating a neutralizing effect of haplogroups K and U on the risk of APOEε4 gene status (Carrieri et al., 2001). The non-random distribution of mitochondrial haplogroups associated with APOEε4 status and the neutralizing effect of mitochondrial haplogroup K on APOEε4 were later confirmed by Maruszak et al. (2011).

The second mode is an enabling effect of APOEε4 on mitochondrial genetic variances as risk factors for AD (see **Table 6**). In non-APOEε4 carriers, SNP mt4336C (a defining SNP for subhaplogroup H5a) was not an AD risk factor, however, in APOEε4 carriers, the same SNP was a risk factor for AD (Edland et al., 2002). This study indicated that APOE genotype could explain the earlier disparity regarding the association between SNP mt4336C and AD (Shoffner et al., 1993; Hutchin and Cortopassi, 1995; Wragg et al., 1995; Zsurka et al., 1998).

A synergistic effect was also observed between APOEε4 and mitochondrial haplotypes (see **Table 6**). For example, SNP mt7028C, a defining SNP for haplogroup H, and subhaplogroup H5 were suggested to act synergistically with APOEε4 to increase risk for AD (Coto et al., 2011; Maruszak et al., 2011).

As with the association between mitochondrial haplotype and the risk of late onset AD, the interaction between APOEε4 and mitochondrial genetic variances in modulating the risk of late onset AD remains debatable. Multiple studies failed to identify any correlation between mitochondrial haplogroup and APOEε4 status or failed to identify an interaction between the two on the risk of developing AD (Zsurka et al., 1998; Chinnery et al., 2000; van der Walt et al., 2005; Mancuso et al., 2007; Lakatos et al., 2010; Santoro et al., 2010; Ridge et al., 2013). Collectively, these disparate findings on the association between mitochondrial

TABLE 7 | Sex differentiates effects of mitochondrial haplogroups on risk of AD.

Haplogroups/SNPs	Female	Male	Authors
H	Increased risk	No effect	Maruszak et al. (2009, 2011)
H5	Increased risk	No effect	Santoro et al. (2010)
HV	Increased risk	No effect	Maruszak et al. (2009, 2011)
T	Decreased risk	No effect	Maruszak et al. (2011)
JT	Decreased risk	No effect	Maruszak et al. (2011)
U	Decreased risk	Increased risk	van der Walt et al. (2004)
KU	No effect	Decreased risk	Maruszak et al. (2011)
mt7028C (H)	Increased risk	No effect	Maruszak et al. (2009)
mt13368A (T)	Decreased risk	No effect	Maruszak et al. (2011)
mt13708G (non-J)	No effect	Increased risk	Maruszak et al. (2009)
mt9055A (K)	No effect	Decreased risk	Maruszak et al. (2011)

Haplogroups and SNPs identified to have sex differences from literatures are listed. A possible haplogroup defined by the SNPs listed is indicated in the parenthesis following the SNP.

haplotype, APOE genotype, and risk of AD emphasize the importance of a precision medicine approach that considers mitochondrial genetic variance in combination with nuclear genetics.

MITOCHONDRIAL HAPLOGROUP AND SEX DIFFERENCES AND RISK OF ALZHEIMER'S DISEASE

Females are at greater lifetime risk for LOAD, and also have higher prevalence and incidence rate than all age-matched males (Brookmeyer et al., 1998; Mielke et al., 2014; Grimm et al., 2016). The higher risk for female is also evident in faster disease progression and greater burden of AD pathology (Aguero-Torres et al., 1998; Corder et al., 2004; Barnes et al., 2005; Skup et al., 2011; Kelly et al., 2013; Mielke et al., 2014; Grimm et al., 2016). While the underlying mechanism remains to be elucidated, increased mitochondrial oxidative stress may play a role (Schuessel et al., 2004; Mandal et al., 2012). Given the effect of mitochondrial genetic variances on mitochondrial function and risk of AD, it is of interest to identify any interaction between mitochondrial haplotypes and sex difference on risk of LOAD.

While almost all the reviewed studies recognized sex differences on risk of LOAD, only a few studied the effects of mitochondrial genetic variances in each sex (van der Walt et al., 2004; Maruszak et al., 2009, 2011; Santoro et al., 2010; see **Table 7**). Within these studies, some mitochondrial genetic variances were found to be associated with AD in females only (van der Walt et al., 2004; Maruszak et al., 2009, 2011; Santoro et al., 2010). Superhaplogroup JT, haplogroup T, a haplogroup T defining SNP mt13368A, a haplogroup U defining SNP 12308G and a non-H defining SNP mt7028T were found to exert protective effects only in females (van der Walt et al., 2004; Maruszak et al., 2011; see **Table 7**). Superhaplogroup HV, haplogroup H, subhaplogroup H5 and a haplogroup H defining SNP mt7028C were identified as risk factors for only in females (Maruszak et al., 2009, 2011; Santoro et al., 2010; see **Table 7**).

In contrast, some variances affected only males (see **Table 7**). Superhaplogroup UK, and SNP mt9055A, a defining SNP for haplogroup K, were found to be associated with reduced risk of AD in males, while SNP mt13708G (for many non-J haplogroups), and SNP mt10398A, a defining SNP for some subhaplogroups of U, were associated with increased risk only in males (van der Walt et al., 2004; Maruszak et al., 2009, 2011; see **Table 7**). Certain mitochondrial genetic variances also showed opposite effects in each sex. For example, haplogroup U was associated with increased risk in males but decreased risk in females (van der Walt et al., 2004; see **Table 7**).

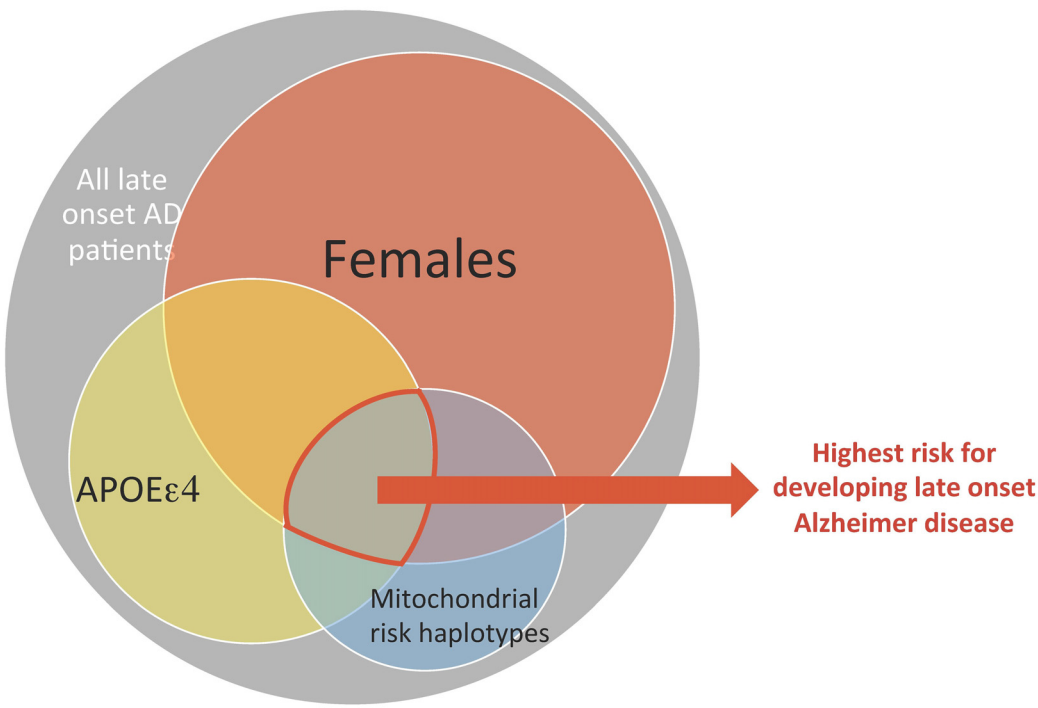

FIGURE 3 | A conceptual framework where the combination of mitochondrial and nuclear genetic risk factors plus sex as a higher risk factor for LOAD. Each of the three factors: sex, APOEε4 genotype and mitochondrial genetic variance has moderate risk for developing late onset AD, but constitutes a much higher risk when combined.

These results indicate that previously observed associations between mitochondrial haplogroups and AD could be driven by sex. Conversely, the effects observed in one sex could be diluted in the whole population, or be neutralized by the other sex, resulting in non-significant associations.

CONCLUSION AND PERSPECTIVES

Mounting evidence suggested a central role of mitochondrial dysfunction in the etiology of late onset AD. As reviewed above, disruption of mitochondrial bioenergetics, structure and dynamics have all been indicated in AD patients. Given the maternal pattern of inheritance of late onset AD and mitochondrial genome, herein we reviewed the association of mitochondrial genetic variances on bioenergetics, respiratory phenotypes and risk of developing LOAD. While the outcomes remain debatable, a large body of science supports an association between mitochondrial genetic variances and differences in bioenergetics and AD susceptibility. Several factors can help explain the disagreement. First, although most studies were conducted in descendants of European origin, the geographic distribution of participants were drastically different, which could result in diverse nuclear genetic background. Since mitochondria communicate extensively with the nucleus, uncontrolled nuclear background can potentially mask effects of mitochondrial genetic variances. Second, as we have reviewed, observations could be driven by certain subhaplogroups, thus results obtained from different populations may be biased by their dominant subhaplogroup. Third, given the heteroplasmic nature of the mitochondrial DNA, accumulated mutations throughout aging may be haplotype and tissue specific (Wallace, 2005), which could contribute to the discrepancies between studies that had different sources of mtDNA (e.g., brain tissues vs. peripheral blood samples; Wallace, 1994). And last, the criteria for mitochondrial haplogroup assignment evolved during the past two decades. Initial studies used haplotype assignment based on only 10 SNPs in the control region, whereas more recent studies used 138 SNPs across the whole human mitochondrial genome. As a result, some ambiguous assignments were resolved and more subhaplogroups were identified.

The data also indicate that mitochondrial haplotype is one factor among several that impacts risk of AD. This is consistent with the multifactorial nature of aging trajectories and risk for LOAD. A systems biology approach that integrates mitochondrial genetic variances and risk factors such as APOEε4 genotype and sex is a step towards resolving disparities across studies of mitochondrial haplotype and risk of neurodegenerative diseases associated with mitochondrial dysfunction (see **Figure 3**). More importantly, we propose a precision medicine approach, where nuclear genetic risk factors (especially APOEε4 genotype), mitochondrial haplotypes, and sex differences can be incorporated into future therapeutic designs for LOAD.

AUTHOR CONTRIBUTIONS

YW and RDB wrote and reviewed the manuscript together.

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REVIEW
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Implications of mitochondrial dynamics on neurodegeneration and on hypothalamic dysfunction

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Mitochondrial dynamics is a term that encompasses the movement of mitochondria along the cytoskeleton, regulation of their architecture, and connectivity mediated by tethering and fusion/fission. The importance of these events in cell physiology and pathology has been partially unraveled with the identification of the genes responsible for the catalysis of mitochondrial fusion and fission. Mutations in two mitochondrial fusion genes (*MFN2* and *OPA1*) cause neurodegenerative diseases, namely Charcot-Marie Tooth type 2A and autosomal dominant optic atrophy (ADOA). Alterations in mitochondrial dynamics may be involved in the pathophysiology of prevalent neurodegenerative conditions. Moreover, impairment of the activity of mitochondrial fusion proteins dysregulates the function of hypothalamic neurons, leading to alterations in food intake and in energy homeostasis. Here we review selected findings in the field of mitochondrial dynamics and their relevance for neurodegeneration and hypothalamic dysfunction.

Keywords: mitochondrial fission, mitochondrial fusion, mitofusin 2, obesity, food intake, neurodegenerative diseases

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Introduction

Mitochondrial filaments were first reported in the mid-1800s upon the discovery of mitochondria. However, it was not until the 1990s that the improvement of mitochondrial dyes permitted the visualization of mitochondrial motion in different cellular models (Bereiter-Hahn and Voth, 1994; Nunnari et al., 1997; Cortese et al., 1998). Mitochondrial dynamics alludes to the movement of mitochondria along the cytoskeleton and also to changes in mitochondrial morphology, both parameters being controlled by fusion and fission events, which in turn lead to these organelles forming tubular or branched reticular networks.

The discovery of the first gene that participates in mitochondria fusion in *Drosophila melanogaster* came about in 1997 (Hales and Fuller, 1997). In the last years, genes that participate in mitochondrial fusion or fission have been identified (Yaffe, 1999). Although considerable advances have been made in the field of mitochondrial dynamics in recent years, the molecular elements that regulate fusion and fission remain unclear. In this regard, the physiological relevance of mitochondrial dynamics in mammals is not precisely understood, and the proteins that determine differences in mitochondrial morphology among distinct cell types need to be clarified.

Mitochondrial dynamics is required to facilitate inter-mitochondrial complementation, and it also participates in ensuring that mitochondria undergo maximal hyperfusion under conditions of cellular stress (Chen and Chan, 2005; Tondera et al., 2009). The fragmentation of mitochondria is also crucial in order for these organelles to undergo mitophagy (Twig et al., 2008), and balanced mitochondrial dynamics is key to the maintenance of an appropriate cell metabolism (Bach et al., 2003; Sebastian et al., 2012).

Major Processes and Proteins Involved in Mitochondrial Dynamics

Here we will review the proteins that participate in mitochondrial fusion and mitochondrial fission.

The Mitochondrial Fusion Machinery

Mitochondrial compartmentalization is ensured by fusion of the inner and outer mitochondrial membranes (Meeusen et al., 2004). The main proteins involved in this process are the outer membrane GTPases Mitofusins (Mfn1 and Mfn2) (Chen et al., 2003; Ishihara et al., 2004) and the inner membrane GTPase Optic atrophy 1 (OPA1) (Cipolat et al., 2004; Ishihara et al., 2006).

Mfn1 and Mfn2 are integral outer mitochondrial membrane proteins and modulate mitochondrial morphology by promoting mitochondria tethering and fusion (Koshiba et al., 2004). The functions of Mfn1 and Mfn2 seems to overlap, since Mfn1 partially rescues the defects caused by Mfn2 mutation (Detmer and Chan, 2007). *Mfn1* and *Mfn2* genes are widely expressed. Mfn1 gene expression is high in heart and is expressed at lower level in other human tissues. Mfn2 transcripts are abundant in heart and skeletal muscle and present at lower levels in other tissues (Santel et al., 2003).

Mfn1 shows two transmembrane domains at the C-terminus of the protein, near a heptad-repeat (HR) domain (Santel et al., 2003). The N-terminal region of Mfn1 contains a GTP-binding domain followed by a heptad-repeat domain (HR1) (Santel et al., 2003; Koshiba et al., 2004). The C-terminal HR domain is considered to mediate the first step of mitochondrial fusion, which consists of the tethering of two adjacent mitochondria through the formation of a dimeric antiparallel coiled-coil structure (Koshiba et al., 2004). These dimeric structures can be homotypic (Mfn1-Mfn1 or Mfn2-Mfn2) or heterotypic (Mfn1-Mfn2) (Chen et al., 2003; Koshiba et al., 2004). In addition, Mfn1 shows higher GTPase activity than Mfn2 (Ishihara et al., 2004). In this respect, mitochondria containing Mfn1 show greater tethering efficiency than mitochondria with Mfn2 (Ishihara et al., 2004).

Mfn1 shows both transcriptional and post-transcriptional or post-translational regulation (Santel et al., 2003). In this regard, Mfn1 is regulated by PGC-1α during postnatal cardiac growth (Martin et al., 2014). In contrast, Mfn1 is repressed by dexamethasome in liver and in hepatoma cells (Hernandez-Alvarez et al., 2013) and by microRNA 140 in cardiomyocytes (Li et al., 2014). Regarding post-translational regulation, Mfn1 undergoes ubiquitination mediated by MARCH-V (also named MITOL) or Parkin (Gegg et al., 2010; Park et al., 2010; Tanaka et al., 2010; Park and Cho, 2012), and deacetylation and activation mediated by HDAC6 (Lee et al., 2014). Mfn1 may be regulated through binding to MIB, a member of the quinone oxidoreductase subfamily of zinc-containing alcohol dehydrogenase proteins (Eura et al., 2006), and overexpression of MIB induces mitochondrial fragmentation, whereas MIB knockdown causes enhanced mitochondrial network structures (Eura et al., 2006).

As mentioned, Mfn2 is an integral outer mitochondrial membrane protein, which exposes both terminal ends to the cytosol (Rojo et al., 2002). The N-terminal GTPase activity of Mfn2 is key for its function in mitochondrial fusion (Chen et al., 2003; Eura et al., 2003). Mfn2 is essential for embryonic development, and ablation of Mfn2 causes placental dysfunction (Chen et al., 2003). Mfn2 exerts a key role in brain, and protects against neurodegeneration in the cerebellum (Chen et al., 2007) as well as in dopaminergic neurons (Lee et al., 2012; Pham et al., 2012). In addition, Mfn2 repression in neurons leads to a delayed cell death upon excitotoxicity (Martorell-Riera et al., 2014). Mfn2 has been proposed to regulate cell proliferation and mitochondrial metabolism (Bach et al., 2003; Chen et al., 2004a, 2014; Pich et al., 2005). In addition, this protein promotes insulin signaling, and deficiency in muscle or liver causes impaired insulin signaling caused by excessive JNK activity and phosphorylation of IRS proteins at serine residues—the latter inhibiting the capacity to activate PI-3 kinase and downstream elements of the pathway (Sebastian et al., 2012). Whether Mfn2 modulates insulin signaling in other tissues such as brain, remains unknown.

The transcription factor Sp1 binds to and drives Mfn2 gene transcription both in skeletal muscle and in smooth muscle cells (Sorianello et al., 2012). The transcription factor Estrogen-Related Receptor-alpha (ERRα) also binds to the human Mfn2 promoter and stimulates transcription (Soriano et al., 2006). The transcription of Mfn2 is stimulated by the coactivators PGC-1α and PGC-1β—key factors in mitochondrial biogenesis (Scarpulla et al., 2012)— through physical/functional interaction with ERRα (Soriano et al., 2006; Liesa et al., 2008). Thus, PGC-1α or PGC1β overexpression induces Mfn2 in cells (Soriano et al., 2006; Liesa et al., 2008). PGC-1β is also necessary for the maintenance of Mfn2 in tissues, and PGC-1β KO mice show reduced Mfn2 in metabolically-relevant tissues (Liesa et al., 2008). In keeping with this, PGC-1β influences mitochondrial morphology through the promotion of mitochondrial elongation, and enhanced mitochondrial fusion (Liesa et al., 2008). Interestingly. overexpression of PGC-1β causes mitochondrial elongation in wild-type, and in Mfn1 KO MEF cells. However, such overexpression does not cause mitochondrial elongation in Mfn2 KO MEF cells (Liesa et al., 2008), which suggests that Mfn2 is required for the effects of PGC-1βon mitochondrial morphology. These data are also consistent with the reduced mitochondrial volume, independent of changes in mitochondrial number, detected in muscle from PGC-1β KO mice (Lelliott et al., 2006; Liesa et al., 2008). Based on all those evidences, Mfn2 undergoes transcriptional regulation.

In addition to this mechanism of control, Mfn2 is regulateded by proteasomal degradation. In this respect, Mfn2 undergoes

ubiquitination catalyzed by Parkin (Gegg et al., 2010; Poole et al., 2010; Tanaka et al., 2010; Ziviani et al., 2010). Mfn2 is also ubiquitinated by the E3 ubiquitin ligases HUWE1, regulated by phosphorylation of JNK, and by the ubiquitin ligase Mul1 (Leboucher et al., 2012; Lokireddy et al., 2012). The processes of Mfn2 ubiquitination lead to Mfn2 degradation. The ubiquitin ligase MARCH-V (or MITOL) catalyzes lysine-63-linked polyubiquitination of Mfn2 but does not drive its proteasomal degradation (Sugiura et al., 2013). Mfn2 levels are also regulated by activation of the deubiquitinase USP30 (Yue et al., 2014).

Optic atrophy gene 1 (OPA1) is also a dynamin-related protein with GTPase activity. This protein is located in the intermembrane space, in the inner mitochondrial membrane and in the cristae volume (Misaka et al., 2002; Olichon et al., 2002; Satoh et al., 2003). OPA1 is essential for mitochondrial fusion (Cipolat et al., 2004; Chen et al., 2005). OPA1 shows high expression in brain, retina, liver, heart, skeletal muscle, and testis (Delettre et al., 2001; Cipolat et al., 2004). OPA1 maintains the normal structure of the optic nerve (Davies et al., 2007), and its deficiency causes alterations in the dendritic morphology of retinal ganglion cells (Williams et al., 2010). In addition, OPA1 promotes neuronal survival following excitotoxicity (Jahani-Asl et al., 2011).

Various OPA1 isoforms have been identified (Olichon et al., 2003; Cipolat et al., 2004; Griparic et al., 2004; Ishihara et al., 2006). The existence of multiple OPA1 isoforms and cleavage mechanisms may explain the role of this protein beyond mitochondrial inner membrane fusion, such as in cristae remodeling, supercomplex formation, and in the regulation of the selective fusion that determines mitochondrial autophagy (Frezza et al., 2006; Twig et al., 2008; Cogliati et al., 2013). OPA1 isoforms are generated from a single gene through alternative splicing and proteolysis. OPA1 isoforms are named as long and short on the basis of their electrophoretic mobility (Delettre et al., 2001; Olichon et al., 2007; Akepati et al., 2008). Both short and long forms are required for mitochondrial fusion, and thus constitutive OPA1 cleavage is required for efficient mitochondrial fusion (Song et al., 2007; DeVay et al., 2009). Some long OPA1 isoforms are constitutively cleaved by the intermembrane space AAA protease YME1L (Griparic et al., 2007; Song et al., 2007). The cleavage of OPA1 is also induced by mitochondrial depolarization, which reduces the abundance of long isoforms, increases the short OPA1 isoforms, and reduces mitochondrial fusion. This inducible OPA1 cleavage is also activated by ATP deficiency and by apoptosis (Baricault et al., 2007; Griparic et al., 2007) and it is catalyzed by the zinc metalloprotease OMA1 (Ehses et al., 2009; Head et al., 2009; Quiros et al., 2012). In this respect, OMA1 ablation causes a deficient mitochondrial activity and function in brown adipose tissue, and obesity (Quiros et al., 2012).

The Mitochondrial Fission Machinery

Mitochondrial fission describes the fragmentation of a mitochondrion into two. This process is necessary to drive damaged mitochondria through mitophagy (Kim et al., 2007), to pass mitochondria to daughter cells during mitosis, and to regulate apoptosis (Lee et al., 2004). Alterations in fission machinery increase the generation of reactive oxygen species (ROS) and lead to a heterogeneous population of mitochondria with non-uniform mitochondrial DNA distribution (Parone et al., 2008).

Dynamin-related protein 1 (Drp1) plays a key role in the catalysis of mitochondrial fission. Drp1 is a cytosolic protein that is recruited to the outer mitochondrial membrane, where it catalyzes mitochondrial division. Drp1 interacts with the mitochondrial proteins fission protein 1 homolog (Fis1), the mitochondrial fission factor (Mff), MiD49, and MiD51.

Drp1 is found mainly in the cytosol and, as a member of the dynamin protein family, it contains a GTPase domain and a GTPase effector domain. Drp1 assembles into multimeric ring complexes at mitochondrial fission sites, which leads to constriction following GTP hydrolysis to promote division (Bleazard et al., 1999; Van Der Bliek, 1999; Legesse-Miller et al., 2003; Ingerman et al., 2005; Lackner et al., 2009; Mears et al., 2011). Given that Drp 1 lacks domains involved in membrane binding, the recruitment of this molecule at the mitochondrial outer membrane requires the involvement of membrane proteins that act as receptors (Parone et al., 2008).

Drp1 is regulated by post-translational modifications such as S-nitrosylation, sumoylation, ubiquitination, and phosphorylation of serine residues, and they may regulate its recruitment to the mitochondria. S-nitrosylation enhances the pro-fission activity of Drp1 by inducing dimerization and enhancing GTPase activity (Cho et al., 2009). Drp1 S-nitrosylation and mitochondrial fragmentation have been detected by the ß-amyloid protein (Aβ), a mediator of Alzheimer's disease (AD) (Head et al., 2009). Drp1 can be also activated by sumoylation (Wasiak et al., 2007). Thus, SUMO-1 and its conjugating enzyme Ubc9 induce mitochondrial fission by stabilizing Drp1 (Harder et al., 2004). In contrast, the sentrin/SUMO-specific protease SENP5 reduces Drp1 levels, which reduces mitochondrial fission (Zunino et al., 2009). In addition, it has been proposed that MARCH V, a mitochondrial E3 ubiquitin ligase, participates in the translocation of Drp1 to mitochondria independently of changes in stability (Karbowski et al., 2007). Phosphorylation occurs on Ser616 and Ser637 (in reference to the human Drp1 sequence). Phosphorylation of Ser616 by cyclin-dependent kinase 1 (Cdk1/cyclin B) causes Drp1 recruitment to the mitochondria (Taguchi et al., 2007). Phosphorylation of Ser637 is catalyzed by protein kinase A (PKA), Calmodulin-dependent kinase, and Pim1 (Chang and Blackstone, 2007; Cribbs and Strack, 2007), and it inhibits Drp1 function. Dephosphorylation of the Ser637 residue by the protein phosphatase calcineurin recruits Drp1 to the mitochondria and promotes fission (Cribbs and Strack, 2007; Cereghetti et al., 2008).

Drp1 ablation in mice causes embryonic lethality. Drp1 knockout embryos present alterations in liver and heart development, increased apoptosis within the deep neural cortex, and deficient synapse formation (Ishihara et al., 2009). In addition, Drp1 loss-of-function delays cytochrome c release during apoptosis, thereby suggesting that this release is linked to mitochondrial fission (Ishihara et al., 2009).

The recruitment of the Drp1 ortholog in yeast (Dnm1) from the cytosol to the outer mitochondrial membrane occurs through association with the protein Fis1, resulting in the formation of a fission complex (Mozdy et al., 2000; Legesse-Miller et al., 2003; Yoon et al., 2003; Karren et al., 2005). Mammalian Fis1 is a small ubiquitous 17.2-kDa protein found throughout the mitochondrial network. Fis1 is anchored into the outer mitochondrial membrane via its COOH terminal part, which contains an alpha-helix, a transmembrane domain, and a COOH-terminal tail exposed to the inter-membrane space. The NH2-terminal part of the protein contains four distinct regions with five alpha-helices (Suzuki et al., 2003; Dohm et al., 2004); the first alpha-helix of Fis1 has been reported to be critical for its oligomerization and fission activity (Jofuku et al., 2005). The subsequent four alpha-helices make up two tetratrico-peptide repeat peptides, which are not required for Fis1 oligomerization but they participate in the protein-protein interactions required for fission (Jofuku et al., 2005). Overexpression of Fis1 causes mitochondrial fragmentation, whereas knockdown of this protein results in the formation of a highly fused mitochondrial network, thereby indicating that Fis1 activates mitochondrial fission (Mozdy et al., 2000; Yoon et al., 2003; Stojanovski et al., 2004; Karren et al., 2005). However, the observations that Drp1 can still be recruited to the mitochondrial outer membrane following knockdown of Fis1 (Lee et al., 2004; Wasiak et al., 2007) suggest that other proteins also participate in mammalian mitochondrial fission.

Mff (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010) is anchored to the outer mitochondrial membrane and it can recruit Drp1 independently of Fis1 (Otera et al., 2010). Absent in yeast, Mff represents a more recent acquisition of the mitochondrial fission machinery that is active in mammalian cells. Mff localizes in discrete sites on mitochondria, and its overexpression causes recruitment of Drp1 to mitochondria and mitochondrial fragmentation. Conversely, Mff deficiency leads to reduced Drp1 at the mitochondrial and to mitochondrial elongation (Otera et al., 2010).

MiD49 and MiD51 proteins share 65% identity and are anchored to the mitochondrial outer membrane through their N-terminal end (Simpson et al., 2000; Palmer et al., 2011). Like Drp1, MiD49/51 form puncta and rings around mitochondria. MiD49/51 recruit Drp1 to the mitochondria, whereas their loss-of-function reduces mitochondrial Drp1 association. Co-immunoprecipitation experiments have established that MiD49 interacts with Drp1. MiD49 and MiD51 can act independently of both Mff and Fis 1 (Loson et al., 2013).

Located in the outer mitochondrial membrane, ganglioside-induced differentiation-associated protein 1 (GDAP1) has been proposed to participate in mitochondrial fission (Cassereau et al., 2011). GDAP1 gain-of-function causes mitochondrial fragmentation, while its deficiency results in mitochondrial elongation (Niemann et al., 2005). Genetic manipulation of the *Drosophila* ortholog Gdap1 also leads to changes in the size, morphology and distribution of mitochondria and in neuronal and muscular degeneration (Lopez Del Amo et al., 2015).

Interaction of the Endoplasmic Reticulum and Mitochondria: Role of Proteins Involved in Mitochondrial Dynamics

Close appositions between the endoplasmic reticulum (ER) and mitochondria or mitochondria-ER contact sites have been observed by electron microscopy in fixed samples of several cell types and are unexplainable by fixation artifacts. These regions represent the sites of phospholipid exchange between the two organelles. The close contacts through which the ER communicates with mitochondria have been biochemically purified and are referred to as the mitochondria-associated ER membrane (MAM) (Vance, 1990). The interaction between mitochondria and the ER is crucial in mediating organelle-organelle signals, such as metabolic stress or cell death cues (**Figure 1**).

Mitochondria tethering to the ER via the MAM is a dynamic process. These ER-contiguous membranes contain multiple phospholipid- and glycosphingolipid-synthesizing enzymes, including long-chain fatty acid-CoA ligase type 4 (FACL4) and phosphatidylserine synthase-1, and they support the direct transfer of lipids between the ER and mitochondria (Stone and Vance, 2000; Cardenas et al., 2010). In addition to supporting lipid transfer, MAMs also exchange Ca^{2+} ions, which regulate processes such as ER chaperone-assisted folding of newly synthesized proteins and enzymes with dehydrogenase activity (Berridge, 2002). In this regard, the truncated variant of the sarcoendoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1T) is induced during ER stress, promotes the transfer of calcium from the ER to mitochondria, and induces apoptosis (Chami et al., 2008). Nearly 30 proteins that play relevant roles in organelle homeostasis are found in this compartment. Furthermore, proteomic studies of mouse brain MAM fractions have identified about 1000 proteins localized in MAMs (Poston et al., 2013). Chaperones, ion channels, proteins of mitochondrial dynamics, and metabolic enzymes are among the molecules detected in MAMs. Thus, the chaperone GRP-75 links the outer mitochondrial membrane protein VDAC with the N-terminus of the inositol 1,4,5-trisphosphate receptor (IP3R), and GRP-75 deficiency reduces calcium transfer from the ER to mitochondria (Szabadkai et al., 2006) (**Figure 2**). Another MAM-localized protein is the Sigma-1 receptor chaperone. Sigma-1 binds GRP-78 and its overexpression counteracts the ER stress response and prevents apoptosis mediated by the release of ER calcium (Hayashi and Su, 2007). PACS2, another protein localized in MAMs, is involved in the ER-mitochondria interaction. PACS2 silencing causes mitochondria and ER fragmentation and also ER stress characterized by enhanced GRP-78 expression and reduced apoptosis (Simmen et al., 2005). The ER protein Nogo also alters the ER-mitochondria unit (Sutendra et al., 2011).

Mfn2 was initially identified as a mitochondria fusion protein. However, it also plays a critical role in maintaining ER morphology by tethering mitochondria and the ER (de Brito and Scorrano, 2008) (**Figure 3**). *Mfn2* null mouse embryonic fibroblasts (MEFs) show a fragmented ER network, and

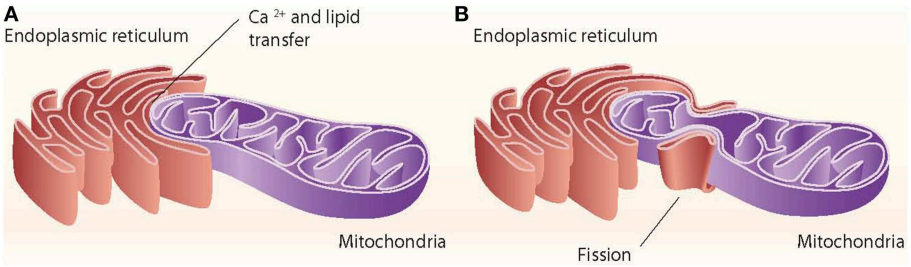

FIGURE 1 | ER–mitochondria contact sites. The ER interacts with mitochondria through close contacts that permit the transfer of calcium from the ER to mitochondria and that permit the exchange of lipids between those two organelles **(A)**. The ER also interacts with mitochondria through mitochondrial fission sites **(B)**.

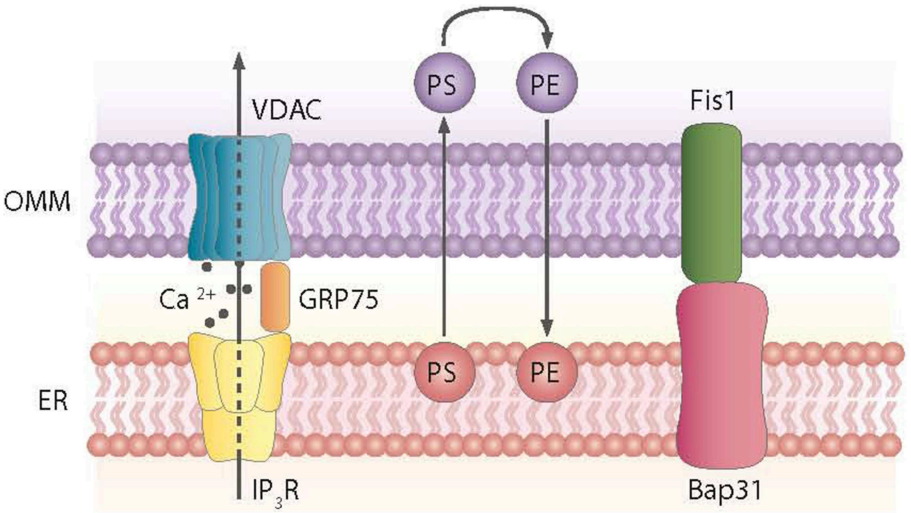

FIGURE 2 | Proteins and processes involved in the ER-mitochondria contact sites. Contact sites involve, among others, the exchange of phosphatidylserine (PS) and phosphatidylethanolamine (PE) between the ER and mitochondria and the formation of complexes between the ER and mitochondrial proteins, as shown.

the contact regions between mitochondria and the ER are significantly reduced (de Brito and Scorrano, 2008). In keeping with this view, Mfn2 loss-of-function in cells or in tissues such as muscle, liver or hypothalamus causes ER stress (Sebastian et al., 2012; Munoz et al., 2013; Schneeberger et al., 2013). Moreover, Mfn2 deficiency causes chronic activation of PERK, a process that plays a relevant role in the alterations detected under these conditions (**Figure 3**). Thus, PERK deficiency in Mfn2 null MEFs reduces ROS production, normalizes mitochondrial calcium, and improves mitochondrial morphology (Munoz et al., 2013).

Other proteins involved in mitochondrial dynamics, such as Drp1 and Fis1, are also associated with ER-mitochondria contact sites (Pitts et al., 1999; Iwasawa et al., 2011). In mammalian cells, the ER tubules contact the mitochondrial membrane at constriction sites marked by Drp1 and its receptor the Mff protein (Friedman et al., 2011). In fact, Drp1 and Mff are markers for ER-mitochondria contact sites. The ER-mitochondria contact is not disrupted by Drp1 or Mff depletion, thereby suggesting that such interactions are independent of division machinery recruitment (Friedman et al., 2011). Contact with the ER is therefore a feature of mitochondrial fission sites (**Figure 1**). This contact is also maintained after mitochondrial fission.

In all, alterations in the expression of Mfn2 lead to the disruption of ER-mitochondria communications and the ER plays a key role in the localization of mitochondrial fission sites.

Mutations in Genes Involved in Mitochondrial Dynamics Cause Neurodegenerative Disorders

In the nervous system, mitochondria are essential for energy production, calcium regulation, maintenance of plasma membrane potential, protein folding by chaperones, axonal and dendritic transport, and release and re-uptake of neurotransmitters at synapses. In the last years, mitochondrial dynamics has been reported to participate in the pathophysiology of neuronal disorders. In this respect, mutations in genes involved in mitochondrial dynamics have been shown to cause neuronal disorders.

Autosomal Dominant Optic Atrophy

Autosomal dominant optic atrophy (ADOA) has an estimated prevalence ranging from 1:12,000 to 1:50,000 and it is the most

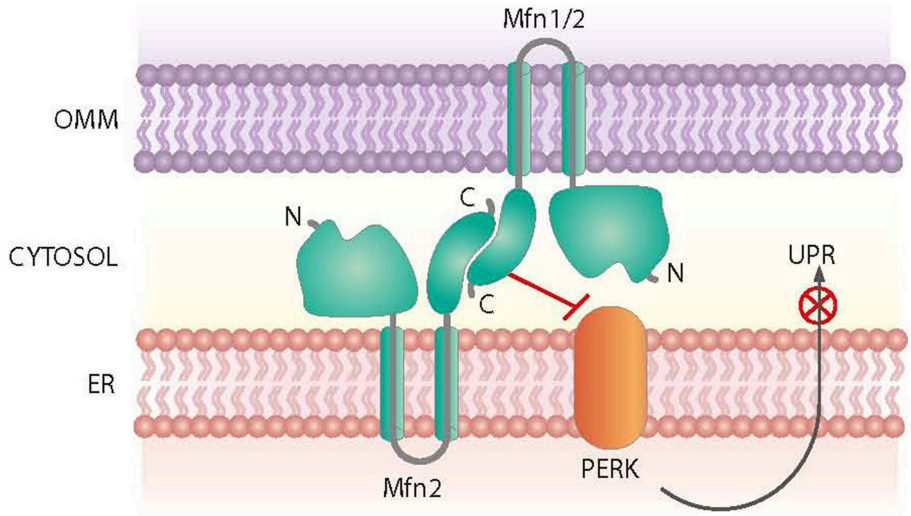

FIGURE 3 | Roles of Mfn2 on ER-mitochondria contact sites. Mfn2 participates in the tethering of mitochondria and the ER at contact sites. In addition, Mfn2 negatively regulates PERK, a protein kinase involved in the Unfolded Protein Response (UPR) and activated upon ER stress.

common form of inherited optic neuropathy. This disease is characterized by visual impairment in early childhood with moderate to severe loss of visual acuity, temporal optic disc pallor, abnormalities of color vision, and caecocentral visual field scotoma (Hoyt, 1980; Votruba et al., 1998; Johnston et al., 1999). Histopathological analysis suggests that the fundamental pathology of ADOA is a primary degeneration of retinal ganglion cells, followed by increasing atrophy of the optic nerve (Johnston et al., 1979; Kjer et al., 1983).

Most families with ADOA show pathogenic mutations in the *OPA1* gene (Olichon et al., 2006). More than 200 OPA1 gene mutations have been identified—these basically family-specific (Ferré et al., 2005). OPA1 mutations involve mainly substitutions, but also deletions and insertions (Delettre et al., 2001; Olichon et al., 2006). Near 50% of the mutations cause truncation of the OPA1 protein, and most of the mutations are detected in the GTPase domain and are thus likely to eliminate mitochondrial fusion. The pathogenesis of ADOA occurs, in most cases, as a result of haploinsufficiency (loss-of-function, Delettre et al., 2000). In this regard, OPA1 deficiency in mice induced by in-frame deletion of 27 amino acid residues in the GTPase domain or non-sense mutations causes the degeneration of retinal ganglion cells and disorganized mitochondrial cristae of optic nerve axons (Alavi et al., 2007; Davies et al., 2007). In addition to haploinsufficiency, ADOA can also develop as a result of a dominant negative mechanism (Delettre et al., 2000; Pesch et al., 2001; Baris et al., 2003; Kim et al., 2005).

Some human OPA1 mutations cause a specific form of disease, characterized by myopathy and progressive external ophtalmoplegia (Hudson et al., 2008), and has been named "OPA1 plus syndrome." This syndrome is also characterized by reduced mitochondrial DNA copy number. In keeping with this, patients with certain OPA1 mutations show multiple deletions in mitochondrial DNA in skeletal muscle (Amati-Bonneau et al., 2008; Hudson et al., 2008), and mice carrying the Opa1(delTTAG) mutation (found in 30% of all human patients with ADOA) show a multi-systemic phenotype (Sarzi et al., 2012).

Mfn2 Mutations: Charcot-Marie-Tooth Type 2A

Charcot-Marie-Tooth (CMT) disease is clinically characterized by weakness and distal muscle atrophy, predominantly of the lower extremities, and by sensory loss. CMT affects approximately 1 in 2500 individuals, thus making this condition one of the most common hereditary diseases and the most common hereditary neuropathy (Skre, 1974).

CMT2 is characterized by chronic axonal degeneration and regeneration, leading to steady loss of nerve fibers with normal or slightly reduced motor nerve conduction velocities ($\geq$38 m/s) (Dyck and Lambert, 1968). Mutations in MFN2 cause 20% of CMT2 cases (Lawson et al., 2005), and this the most prevalent axonal form of CMT. CMT2 cases show high variability in clinical symptoms. Furthermore, MFN2 mutations have been detected in CMT2 families and are associated with additional features such as spasticity (Zhu et al., 2005) and atrophy (Verhoeven et al., 2006).

Most of the MFN2 mutations found in CMT2A patients are missense (Zuchner et al., 2004; Kijima et al., 2005; Lawson et al., 2005; Zhu et al., 2005; Chung et al., 2006; Engelfried et al., 2006; Verhoeven et al., 2006). More than 50% are detected in the GTPase domain but they do not affect GTP binding (Baloh et al., 2007). In addition, MFN2 mutations have been detected in the following sites: in the NH2-terminal region; near or at the Ras-binding domain; in the vicinity of or at the HR1 region; and in the COOH terminus, specifically in the HR2 region, facing the cytoplasmic site.

The mutations of MFN2 responsible for CMT2A show autosomal dominant inheritance; consequently, they may show haploinsufficiency or a dominant gain-of-function. In keeping with this view, the overexpression of some mutant forms of MFN2 induces the aggregation of mitochondria in cultured

rat dorsal root ganglion neurons and in MEFs, indicating the promotion of gain-of-function (Baloh et al., 2007; Detmer and Chan, 2007). The defective mitochondrial fusion activity of some Mfn2 mutants is rescued by Mfn1 gain-of-function, which is in keeping with the observation that Mfn1 physically associates with wild-type Mfn2 and mutant forms of CMT2A (Loiseau et al., 2007; Amiott et al., 2008).

Transgenic mice have been generated to express a mutant form of MFN2 with a presumed gain-of-function (T105M) specifically in motor neurons. These mice show a phenotype consistent with the clinical symptoms detected in CMT2A, and provide a system to determine the function of mitochondria in the axons of motor neurons (Detmer et al., 2008).

GDAP1 mutations are associated with type 4A CMT (CMT4A), the most frequently detected recessive form of CMT (Baxter et al., 2002; Cuesta et al., 2002). CMT4 is classically defined as a demyelinating form of the disease and it is associated with segmental de- and re-myelination, in contrast to CMT2, which shows axonal degeneration without demyelination (Berger et al., 2002; Suter and Scherer, 2003). However, analysis of the nerve conduction rates of subjects with GDAP1 mutations indicates that while some mutations show low nerve conduction rates (Baxter et al., 2002; Ammar et al., 2003; Senderek et al., 2003), others are characterized by normal rates (Nelis et al., 2002; Ammar et al., 2003; Boerkoel et al., 2003; De Sandre-Giovannoli et al., 2003; Sevilla et al., 2003). These data demonstrate that some of the GDAP1 mutations cause axonal loss rather than demyelinization.

Mitochondrial Dynamics and Prevalent Neurodegenerative Diseases

Both fusion and fission mechanisms participate in the mitochondrial life cycle and any disruption of their balance can alter the steady-state distribution of mitochondria. Specifically in neurons, the mitochondrial fusion/fission machinery is intimately and critically involved in the formation of synapses and dendritic spines. Thus, alterations in mitochondrial dynamics prevent these organelles from distributing to synapses, thus leading to a loss of mitochondria from dendritic spines and, consequently, to a reduction of synapse formation (Li et al., 2004). The mitochondrial dynamics proteins that control the distribution of mitochondria in dendrites also regulate the density and plasticity of synapses (Li et al., 2004). Thus, dominant-negative Drp1 or OPA1 overexpression (both of which reduce dendritic mitochondria) causes a decreased density of spines and synapses (Li et al., 2004). Consistent with this view, Drp1 ablation in mice shows developmental abnormalities, particularly in the forebrain (Ishihara et al., 2009). Given these considerations, a strong association between the expression/activity of proteins involved in mitochondrial dynamics and neurodegenerative diseases can be expected.

In this regard, brains from AD patients show fragmented and perinuclear mitochondria (Cho et al., 2009; Wang et al., 2009a), paralleled by increased expression of Fis1 and decreased expression of the fusion proteins Mfn1, Mfn2, and OPA1 (Wang et al., 2009a; Reddy et al., 2011). These data suggest that abnormal mitochondrial dynamics in neurons of AD patients may participate in the pathogenesis of the disease. In line with these observations, brains from AD patients show altered lipid metabolism (Schon and Area-Gomez, 2010), aberrant calcium homeostasis (Supnet and Bezprozvanny, 2010), enhanced unfolded protein response (UPR) (Hoozemans et al., 2005), and defects in energy metabolism (Ferreira et al., 2010). As to the factors responsible for mitochondrial dysfunction, overexpression of amyloid precursor protein (APP) or exposure to amyloid beta peptide (Abeta) induces mitochondrial fragmentation and abnormal distribution (Rui et al., 2006; Wang et al., 2008). Furthermore, exposure of neurons to oligomerized Abeta leads to S-nitrosylation of Drp1, causing mitochondrial fragmentation (Cho et al., 2009). Upregulated MAM function at the ER-mitochondrial interface and increased cross-talk between these two organelles has been reported in presenilin-mutant cells, and it may participate in the pathogenesis of AD (Area-Gomez et al., 2012).

Complex I activity is reduced in the substantia nigra of subjects with Parkinson's disease (PD), and various complex inhibitors (MPP^+, rotenone, and other pesticides) cause neuropathological changes similar to those observed in PD (Schapira et al., 2006). In this regard, rotenone and 6-hydroxydopamine have been shown to induce Drp1-dependent mitochondrial fragmentation in neuronal cells (Barsoum et al., 2006; Gomez-Lazaro et al., 2008). Mutations in Parkin and outer membrane kinase PTEN-induced putative kinase-1 (PINK1) have been reported in familial PD, and these proteins participate in a major mitochondrial quality control pathway (Pickrell and Youle, 2015). When the mitochondrial membrane potential decreases, PINK1 recruits Parkin to the mitochondria (Narendra et al., 2010). Parkin next ubiquitinates PINK1, thus trigering mitophagy. Parkin also ubiquitinates other targets such as Mfn1, Mfn2, Fis1, and Drp1, causing their proteasomal degradation (Tanaka et al., 2010; Ziviani et al., 2010; Chan et al., 2011; Wang et al., 2011). In agreement, loss of function of PINK1 or Parkin causes mitochondrial fragmentation (Exner et al., 2007; Lutz et al., 2009; Sandebring et al., 2009), and mammalian fibroblasts carrying PINK1 mutations from PD patients also exhibit mitochondrial fragmentation (Grunewald et al., 2009).

Huntington's disease (HD) is caused by an expansion of a CAG trinucleotide sequence that encodes a polyglutamine tract in the huntingtin protein. Mitochondrial dysfunction is also associated with the pathogenesis of HD (Brouillet et al., 1995; Tabrizi et al., 1999; Panov et al., 2002; Schapira et al., 2014). Thus, the striatum and frontal cortex of HD patients show an increased abundance of Fis1 and Drp1 (Costa et al., 2010) and decreased levels of Mfn1, Mfn2, and OPA1 (Shirendeb et al., 2011). This pattern of changes may lead to mitochondrial fragmentation in these subjects. As to the mechanisms responsible for mitochondrial fragmentation, overexpression of mutant huntingtin causes mitochondrial fragmentation and blockade of fragmentation prevents the cytotoxic effects of mutant huntingtin (Wang et al., 2009b). Furthermore, mutant huntingtin binds to Drp1 and enhances its GTPase activity (Song et al., 2011). In all, these studies

support the notion that alterations in mitochondrial dynamics are involved in the pathogenesis of HD.

Hypothalamic Neuronal Circuits and Mitochondrial Function

The maintenance of energy homeostasis is fundamental to sustain life. In mammals, the central nervous system (CNS) plays a critical role in the regulation of appetite, energy expenditure, and metabolism through multiple and distributed neuronal circuits. The hypothalamus, which is made up of distinct nuclei, including the arcuate nucleus (ARC), the paraventricular nucleus, the lateral area, the dorsomedial nucleus and the ventromedial nucleus, is arguably the most important CNS region in metabolic control (Schneeberger et al., 2014). Extensive experimental evidence indicates that ARC is a key area in the neural hierarchy that regulates this biological process. The ARC holds at least two subsets of neurons—with opposite functions and reciprocally regulated—involved in systemic energy balance control. One set co-expresses orexigenic neuropeptides Agouti-related protein (AgRP) and Neuropeptide Y (NPY), while the other co-expresses anorexigenic neuropeptides cocaine- and amphetamine-regulated transcript (CART) and α-melanocyte-stimulating hormone (α-MSH, a product of proopiomelanocortin (POMC) processing) (Schneeberger et al., 2014).

The Melanocortin System

POMC and AgRP neurons, together with downstream neurons expressing melanocortin receptors (MCR) 3 and 4, form the melanocortin system, a crucial neuronal circuit that senses and responds to fluctuations in central and circulating factors that inform about the nutritional status of the organism. Indeed, the activity of these two subsets of neurons is influenced by local (NPY, serotonin, GABA, etc.) and peripheral (leptin, insulin, ghrelin) signals, as well as by nutrients such as glucose and free fatty acids. Numerous pharmacological and genetic studies indicate that the divergent physiological roles of these two populations of neurons are largely the consequence of the release and action of α-MSH and AgRP neuropeptides. α-MSH is an endogenous agonist of MCR3 and 4, thus providing an anorexigenic tone by suppressing appetite and increasing thermogenesis (Poggioli et al., 1986; Wirth et al., 2001). In contrast, AgRP is an antagonist of these receptors and therefore counteracts the effects of α-MSH signaling on food intake and body weight (Ollmann et al., 1997). In addition, the orexigenic actions of AgRP neurons are also mediated by the release of NPY, which binds to specific receptors, and by direct GABAergic synapsis onto POMC neurons (Cowley et al., 2001).

In summary, current evidence indicates that a local ARC circuit constituted by "first order" POMC and AgRP neurons plays a critical role in sensing, integrating, and responding to humoral signals that report on energy status. These neurons engage downstream "second order" multi-level neurocircuits that will produce precise effector responses. The global integration of these signals culminates in the generation of coordinated behavioral, autonomic, and neuroendocrine responses to regulate appetite, energy expenditure, and body weight (Schneeberger et al., 2014).

Leptin and Ghrelin: Key Hormones Regulating Energy Homeostasis

Leptin and ghrelin are prototypical examples of metabolic signals of energy surfeit and deficit respectively, and POMC and AgRP neurons are direct targets of both hormones (Cheung et al., 1997; Elias et al., 1999; Willesen et al., 1999; Cowley et al., 2001). Leptin exerts its anorexigenic effects by increasing *Pomc* expression and processing into α-MSH (Schwartz et al., 1997; Thornton et al., 1997; Mizuno et al., 1998), and by inhibiting both *Npy* and *AgRP* transcription (Stephens et al., 1995; Schwartz et al., 1996; Mizuno and Mobbs, 1999). Leptin also increases the electrical activity of POMC neurons, thereby leading to α-MSH release (Cowley et al., 2001; Claret et al., 2007; Hill et al., 2008; Al-Qassab et al., 2009). In addition to its agonism/antagonism effects on MCRs, leptin attenuates the inhibitory GABAergic tone of AgRP neurons onto POMC neurons (Cowley et al., 2001). Similarly, recent data showed that leptin also acts on non-AgRP presynaptic GABAergic neurons by reducing the inhibitory input on POMC neurons (Vong et al., 2011). Overall, these effects result in reduced food intake and increased energy expenditure.

Under conditions of negative energy balance, circulating ghrelin levels are increased. Evidence indicates that the orexigenic effects of ghrelin are largely mediated by AgRP neurons (Chen et al., 2004b; Luquet et al., 2007; Wang et al., 2014). Consistently, ghrelin enhances *Npy* and *AgRP* transcript expression (Kamegai et al., 2001; Nakazato et al., 2001). The release of AgRP and NPY hyperpolarizes neighboring POMC neurons, thus reducing α-MSH release (Roseberry et al., 2004; Smith et al., 2007; Cyr et al., 2013). Furthermore, electrophysiology studies have shown that ghrelin activates AgRP neurons and increases the number of inhibitory synapses onto POMC neurons (Cowley et al., 2003; Yang et al., 2011; Atasoy et al., 2012). Collectively, these events lead to the simultaneous activation of AgRP neurons and inhibition of POMC neurons, thus enhancing the orexigenic output.

Mitochondrial Function in Hypothalamic ARC Neurons

Neurons are highly energy-demanding, and thus adequate mitochondrial performance is essential for the growth, survival, and function of these cells (Zhu et al., 2012). Mitochondrial ATP production and Ca^{2+} buffering/release are fundamental for a myriad of neuronal functions, including synapse assembly, action potential generation, and synaptic transmission, amongst others. Given their particular and intricate morphology, neurons require the distribution of mitochondria to distal areas in order to cover the high energy demands associated with a number of specialized functions. Thus, mitochondrial function and dynamics must be tightly regulated in order to temporally and spatially satisfy the bioenergetic needs of neurons.

Despite the paramount importance of the regulation of systemic energy balance and metabolism by mitochondria in hypothalamic neurons, few studies have addressed this issue. An important aspect to note is the diametrically different

bioenergetic patterns of POMC and AgRP neurons, which are largely the consequence of their divergent physiological functions. Negative energy balance acutely activates AgRP neurons and promotes mitochondrial uncoupling activity associated with elevated mitochondrial density (Coppola et al., 2007; Dietrich et al., 2013). Under these conditions, ghrelin-induced β-oxidation constitutes the main energy supply to maintain AgRP neuron activity. This is achieved through an axis formed by the AMP-activated protein kinase (AMPK) and the muscle isoform of carnitine O-palmitoyltransferase 1 (CPT1-M) (Andrews et al., 2008; Lopez et al., 2008) which allows the uptake of long-chain fatty acyl CoAs by the mitochondria and the subsequent β-oxidation. A key component of this process is uncoupling protein 2 (UCP2), a mitochondrial protein that mediates proton leak, thereby limiting ATP and ROS production (Mailloux and Harper, 2011). Hypothalamic UCP2 expression is induced by fasting (Coppola et al., 2007), and ghrelin-driven feeding by AgRP requires UCP2 (Andrews et al., 2008). In line with these observations, fasting and ghrelin increase mitochondrial proliferation, synaptic plasticity, and electric activity of AgRP neurons in a UCP2-dependent manner (Coppola et al., 2007; Andrews et al., 2008). Under conditions of positive energy balance, when circulating leptin and glucose levels are high, POMC neurons increase their firing rate while AgRP neurons remain silent (Claret et al., 2007; Parton et al., 2007). Consistently, feeding conditions are associated with increased mitochondrial number in POMC neurons (Diano et al., 2011). The current model suggests that cellular glucose metabolism in POMC neurons generates a high proton gradient, concomitant with elevated ATP and ROS production. ATP would inactivate ATP-sensitive potassium (K_{ATP}) channels and depolarize POMC neurons (Ibrahim et al., 2003). In addition, enhanced ROS levels would activate POMC neurons, further contributing to satiety (Diano et al., 2011). Mitochondrial UCP2 may be a key integrator of these processes, as its activity may limit ATP and/or ROS production and thereby modulate neuronal activity.

Collectively, these lines of evidence support the notion that mitochondrial processes and intermediates in ARC hypothalamic neurons play fundamental roles in sensing and integrating metabolic cues and also mediate neuronal function and associated behavior.

Role of Mitochondrial Fusion Proteins on Hypothalamic ARC Neurons

Recent research suggests that mitochondrial dynamics, in addition to constitute a key quality control mechanism, is also implicated in sensing nutrient fluctuations and promoting bioenergetic adaptations to meet the cellular metabolic needs. In general terms, physiological processes associated with enhanced energy demand and reduced energy supply (acute stress, starvation, etc.) are characterized by mitochondrial fusion and coupled respiration. In contrast, physiological situations associated with decreased energy demand and increased supply (high nutrient levels, obesity, type 2 diabetes) correlate with mitochondrial fission and decreased coupling (Liesa and Shirihai, 2013; Gao et al., 2014). Given that hypothalamic POMC and AgRP neurons are metabolic transceivers, it is reasonable to speculate that mitochondrial dynamics is an integral part of the molecular mechanisms by which these neurons sense the energy/nutritional milieu and mediate specific physiological actions to maintain energy homeostasis.

Two recent studies, one from our research group, have investigated mitochondrial dynamics and the role of fusion proteins Mfn1 and 2 in POMC and AgRP neurons upon energy balance control *in vivo* (Schneeberger et al., 2013). Electron microscopy studies under fasting conditions showed decreased mitochondria number but unaltered morphology in POMC neurons, while AgRP neurons exhibited elevated mitochondrial density and reduced size suggesting enhanced fission. High-fat diet (HFD) administration reduced mitochondria number but increased their size and elongation in AgRP neurons, in line with increased fusion (Dietrich et al., 2013). In contrast, POMC neuron mitochondrial elongation and network complexity was reduced, indicating elevated fission (Schneeberger et al., 2013) (**Figure 4**). Together, these results demonstrate specific patterns of mitochondrial dynamics in response to nutrient challenges in POMC and AgRP neurons, which is consistent with their divergent physiological functions and fuel usage.

Remarkably, HFD feeding reduced the number of mitochondria-ER contacts in POMC neurons by ∼50%, a defect

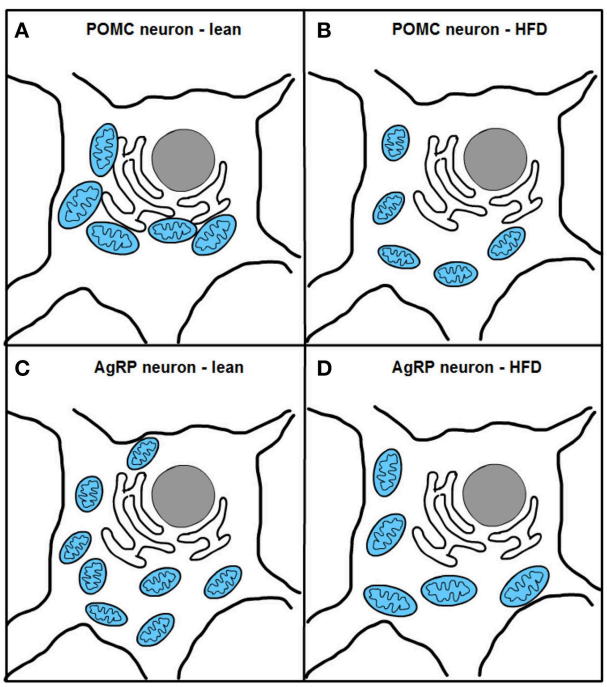

FIGURE 4 | Excess nutrient availability alters ER-mitochondria contacts and mitochondrial dynamics in POMC and AgRP neurons. High-fat diet (HFD) administration increases mitochondrial fragmentation and impairs ER-mitochondria contacts in POMC neurons **(A,B)**. In contrast, AgRP neurons under HFD conditions show increased fusion and unaltered ER-mitochondria contacts **(C,D)**.

that was not observed in the AgRP subpopulation (**Figure 4**). Recent reports have shown that Mfn2 is implicated in the tethering of mitochondria with ER (de Brito and Scorrano, 2008) and in the modulation of ER stress responses (Papanicolaou et al., 2012; Sebastian et al., 2012; Munoz et al., 2013). Indeed, under diet-induced obesity (DIO) conditions hypothalamic Mfn2 expression was reduced and its overexpression in the ARC was able to ameliorate the metabolic disturbances and reduce ER stress markers in the hypothalamus of DIO mice (Schneeberger et al., 2013). Consistent with these observations, conditional deletion of Mfn2 in POMC neurons resulted in a marked obesogenic phenotype as a consequence of loss of mitochondria-ER contacts and early development of ER stress-induced leptin resistance (**Figure 5**). In recent years, hypothalamic ER stress has emerged as a causal factor in the development of leptin resistance and obesity (Zhang et al., 2008; Ozcan et al., 2009) and this study established Mfn2 as a key molecular determinant connecting these processes. Interestingly, the number of mitochondria-ER junctions in POMC neurons may be a potential readout of leptin sensitivity (Schneeberger et al., 2013; Long et al., 2014). The specificity of this dramatic obese phenotype was demonstrated by the lack of energy balance abnormalities in mice lacking the homologous Mfn1 in the same population of neurons (Schneeberger et al., 2013).

Dietrich and associates (Dietrich et al., 2013) also used genetic conditional approaches to investigate the roles of Mfn1 and Mfn2 in AgRP neurons. The metabolic alterations observed in these mouse models were minimal under chow diet conditions (only females showed a slight metabolic improvement). However, a significant observation was that administration of HFD did not lead to the mitochondrial fusion in AgRP-specific knockout mice, suggesting that Mfn1- and Mfn2-mediated fusion was required for the bioenergetic adaptations of this population of neurons to positive energy balance (Schneeberger et al., 2013). This could be the consequence of reduced firing rate and increased number of silent AgRP neurons seen these dietary conditions.

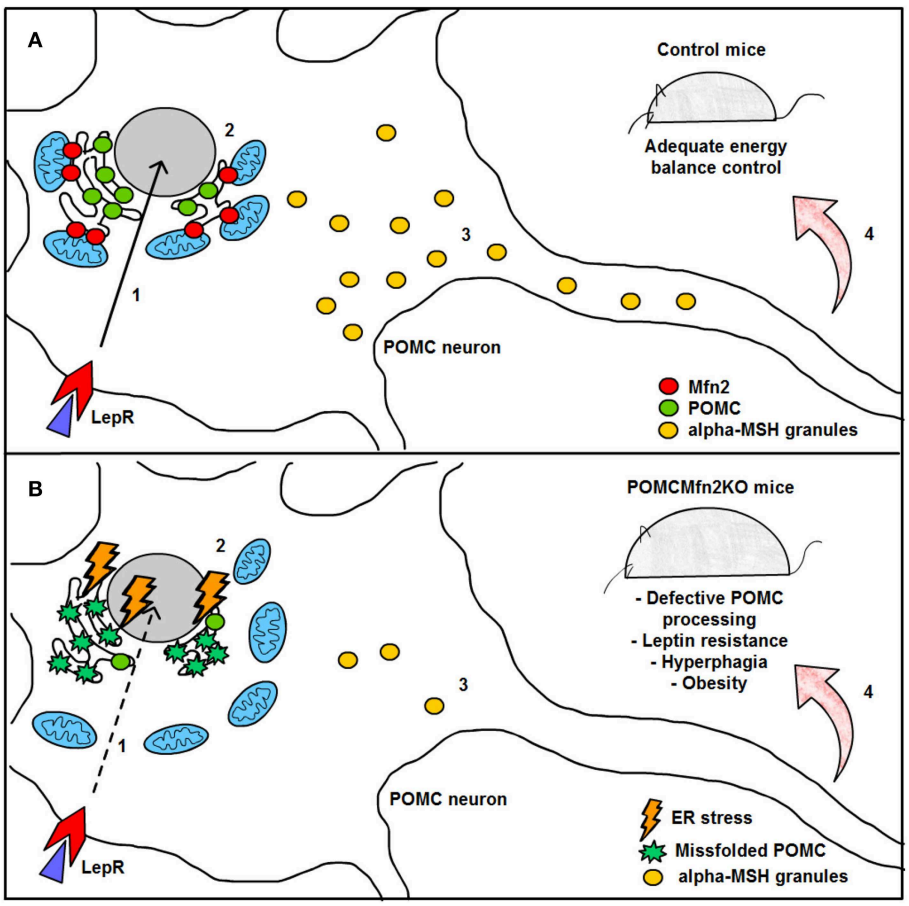

FIGURE 5 | Graphical summary of the consequences of Mfn2 loss in POMC neurons. (A) Under normal physiological conditions, leptin signaling in POMC neurons is adequately transmitted, thereby enhancing POMC transcription in the nucleus (1) and subsequent synthesis in the ER (2). POMC precursor is sorted into secretory granules and processed into α-MSH (3). This neuropeptide is then released into target areas by POMC neuron axonal terminals thus mediating the anorexigenic effects of leptin (4). **(B)** Deletion of Mfn2 specifically in POMC neurons causes loss of ER-mitochondria contacts, thus leading to ER stress which interferes with proper POMC folding (2). Missfolded POMC precursor can not be adequately sorted or processed into α-MSH (3). As a consequence, leptin signaling (1) and leptin-mediated anorexigenic effects are blunted (4). LepR; leptin receptor; Mfn2: mitofusin 2; POMC: proopiomelanocortin; α.MSH: alpha-melanocyte stimulating hormone.

Collectively, and in line with previous studies (Chen et al., 2007; Papanicolaou et al., 2011, 2012; Lee et al., 2012; Pham et al., 2012), these results reinforce the idea of non-overlapping physiological roles for mitofusin proteins and neuron-specific functions. For example, Mfn2 in POMC neurons is particularly involved in maintaining mitochondria-ER contacts and in the modulation of the cellular responses to ER stress, while in AgRP neurons mediates mitochondrial dynamics. It is interesting to note that in other neuronal types, but not in POMC and AgRP neurons, mitofusin proteins are essential for neuron survival and axon growth (Chen et al., 2007; Lee et al., 2012; Pham et al., 2012). The precise description of the basis of such divergent cell-specific biological functions will be of capital importance to understand the role of mitochondrial dynamics in hypothalamic neurons upon the regulation of energy balance and metabolism. These mechanisms will be also important to design potential targeted therapeutical approaches to counteract highly prevalent metabolic disorders such as obesity and type-2 diabetes.

Future Perspectives

A major current medical need is the efficient treatment of prevalent diseases such as obesity, type 2 diabetes, Alzheimer's disease, Parkinson's disease, or Huntington disease. In this respect, it is relevant to identify druggable proteins that participate in the pathophysiology of those disorders, and that permit to stop the progression of the disease or to ameliorate many of the alterations associated to them.

With the turning of the century we have learnt that alterations in genes relevant in mitochondrial dynamics are sufficient to develop diseases, leading to certain forms of CMT and ADOA. Furthermore, data obtained in the last years support the view that alterations in mitochondrial dynamics may be involved in the pathogenesis of neurodegenerative diseases, and in the hypothalamic dysfunctions leading to dysregulated energy balance. Ample evidence indicates that some neurodegenerative disorders are associated with altered metabolic and appetitive behaviors that may be explained by abnormal hypothalamic function. Although it is tempting to speculate that such perturbations are the consequence of defective mitochondrial dynamics, to date direct experimental evidence has not been provided. Certainly, there is a need to fully demonstrate the role of mitochondrial dynamics in the pathogenesis of metabolic and neurodegenerative diseases, and its potential link, by using more sophisticated mouse models and in humans. In spite of these difficulties, we hope that the thorough understanding of the mechanisms that control mitochondrial dynamics, and the interaction between mitochondrial and endoplasmic reticulum will permit to enter into a phase in which some of the mitochondrial dynamics proteins or regulators are amenable for pharmacological manipulation.

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REVIEW
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Microglial cell dysregulation in brain aging and neurodegeneration

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Aging is the main risk factor for neurodegenerative diseases. In aging, microglia undergoes phenotypic changes compatible with their activation. Glial activation can lead to neuroinflammation, which is increasingly accepted as part of the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD). We hypothesize that in aging, aberrant microglia activation leads to a deleterious environment and neurodegeneration. In aged mice, microglia exhibit an increased expression of cytokines and an exacerbated inflammatory response to pathological changes. Whereas LPS increases nitric oxide (NO) secretion in microglia from young mice, induction of reactive oxygen species (ROS) predominates in older mice. Furthermore, there is accumulation of DNA oxidative damage in mitochondria of microglia during aging, and also an increased intracellular ROS production. Increased ROS activates the redox-sensitive nuclear factor *kappa* B, which promotes more neuroinflammation, and can be translated in functional deficits, such as cognitive impairment. Mitochondria-derived ROS and cathepsin B, are also necessary for the microglial cell production of interleukin-1β, a key inflammatory cytokine. Interestingly, whereas the regulatory cytokine TGFβ1 is also increased in the aged brain, neuroinflammation persists. Assessing this apparent contradiction, we have reported that TGFβ1 induction and activation of Smad3 signaling after inflammatory stimulation are reduced in adult mice. Other protective functions, such as phagocytosis, although observed in aged animals, become not inducible by inflammatory stimuli and TGFβ1. Here, we discuss data suggesting that mitochondrial and endolysosomal dysfunction could at least partially mediate age-associated microglial cell changes, and, together with the impairment of the TGFβ1-Smad3 pathway, could result in the reduction of protective activation and the facilitation of cytotoxic activation of microglia, resulting in the promotion of neurodegenerative diseases.

Keywords: Alzheimer's disease, glia, mitochondria, neurodegenerative diseases, neuroinflammation, oxidative stress, reactive oxygen species, transforming growth factor-β

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Introduction

Aging is a complex process of cumulative changes. A key hallmark is the progressive decline in physiological functions and behavioral capacity, which is observed at various levels of the organism, in particular at the central nervous system (CNS; Smith et al., 2005). These changes can lead to altered behavior, memory impairment, or loss of several control functions (Lipsitz and Goldberger, 1992; Lipsitz, 2002; Glenn et al., 2004). In addition, some responses of the immune system, in special

related to adaptive immune system, also decline with age, increasing the susceptibility to infections and cancer. By contrast, other immune responses are exacerbated, facilitating the onset of autoimmune diseases (Yung and Julius, 2008) or the generation of a mild chronic neuroinflammation mediated by the dysregulation of the innate immune system, as will be discussed here. Therefore, aging can affect several tissues and processes, leading to highly complex functional changes.

Microglia undergoes several age-related changes that contribute to the generation of a chronic mild inflammatory environment, including an increased production of inflammatory cytokines and the production of reactive oxygen species (ROS). These changes have been linked to the appearance of cognitive deficits and the onset of chronic neurodegenerative diseases. Therefore, it has been proposed that aging of microglia could contribute to other age-associated brain changes and cognitive decline (Conde and Streit, 2006a,b; Streit, 2006; von Bernhardi, 2010; Aguzzi et al., 2013; Kettenmann et al., 2013).

Normal Brain Aging

Several structural and functional changes associated with normal brain aging have been reported. Brain mass decreases in the order of 2 to 3% per decade after the age of 50. Individuals that are 80 years or older, brain mass is reduced by 10% compared with that of young adults (Drachman, 2006). Magnetic resonance imaging (MRI) and voxel-based morphometry (VBM) show that age specially affects the volume of gray and white matter at prefrontal, parietal, and temporal areas (Ge et al., 2002; Sowell et al., 2003; Salat et al., 2004). Complex learning abilities, such as dual tasks (ea. memorizing a word list while walking), show a progressive decrease during aging (Lindenberger et al., 2000; Salat et al., 2005). Nevertheless, cognitive decline in aging is highly variable; many older people keep intact their cognitive abilities (Shock et al., 1984) until advanced ages.

At the cellular level, shortening of telomeres and activation of tumor suppressor genes, as well as accumulation of DNA damage, oxidative stress, and mild chronic inflammatory activity are characteristic of aging cells. Various tissues, including the brain show an imbalance between pro- and anti-inflammatory cytokine levels. In addition, potentially damaging mediators, such as cytokines, radical species (**Figure 1**), and eicosanoids among others, are produced in response to the exposure to physical, chemical or biological agents, such as ionic radiation, pollutants, pathogens, etc. (Dröge and Schipper, 2007; Vijg and Campisi, 2008). Both humans and mice show decreased levels of interleukin 10 (IL10; Ye and Johnson, 2001), and increased levels of tumor necrosis factor α (TNFα) and IL1β in the CNS (Lukiw, 2004; Streit et al., 2004a), and IL6 in plasma (Ye and Johnson, 2001; Godbout and Johnson, 2004). In addition, increased transforming growth factor β1 (TGFβ1) mRNA a key cytokine regulator, has been observed in the brain of aged mice and rats (Bye et al., 2001).

At the same time, several changes induced by an aged micro-environment, such as increased systemic inflammation, increased permeability of the blood-brain barrier (BBB), and degeneration of neurons and other brain cells, could contribute to the production of ROS. It has been proposed that BBB permeability increases in aged animals (Blau et al., 2012; Enciu et al., 2013), facilitating perhaps infiltration by monocytes releasing mitochondria-generated ROS. An age-related increase in the number of $CD11b^{+}$ $CD45^{high}$ cells, compatible with infiltrated monocytes, has been reported in the brain of aged rats (Blau et al., 2012). Likewise, expression levels of chemotactic molecules, such as interferon-inducible protein 10 (IIP10) and monocyte chemotactic protein-1 (MCP-1), are increased in the hippocampal region (Blau et al., 2012).

Glial Cells, Neuroinflammation and Oxidative Stress

Neuroinflammation is choreographed by microglia and astrocytes, and is defined by increased levels of a complex arrangement of mediators, including IL1β, TNFα and TGFβ, all of which are increased in aged individuals (McGeer and McGeer, 2001; von Bernhardi, 2007; von Bernhardi et al., 2010). Microglia are the brain resident macrophages (Hemmer et al., 2002; Ransohoff and Perry, 2009; Rivest, 2009) providing its first line of defense. In the brain of healthy adults, microglia are slender ramified cells that constantly survey brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005). When stimulated, microglia activate, enlarge their cell body (Nimmerjahn et al., 2005; Frank-Cannon et al., 2009) and change their functional properties (Liu et al., 2001; von Bernhardi and Eugenín, 2004; Lue et al., 2010). Microglia sense and act on a broad range of stimuli, including autoimmune injury, infection, ischemia, toxic insults and trauma (Streit, 2002; Kim and de Vellis, 2005; Schwab and McGeer, 2008; Lue et al., 2010; von Bernhardi et al., 2010). They recognize a broad spectrum of molecular targets, such as glycolipids, lipoproteins, nucleotides, peptides, (Nakamura, 2002; van Rossum and Hanisch, 2004; Pocock and Kettenmann, 2007), abnormally processed, modified or aggregated proteins (e.g., Aβ), inflammatory cytokines, and damaged neurons, which are the strongest inducers of microglia activation (Nakamura, 2002; Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Lue et al., 2010; Schuitemaker et al., 2012). Depending on the stimuli, microglia undergoes different activation patterns (Gordon, 2003; Martinez et al., 2008; Mosser and Edwards, 2008). They include (i) classical M1 activation, which can associate with cytotoxicity, (ii) alternative phagocytic/neuroprotective M2 activation (Gordon, 2003; Martinez et al., 2008) or (iii) regulatory activation (Mosser and Edwards, 2008). Thus, activated microglia show a continuum spectrum of activation patterns, resulting in the expression of different cytokines and cytokine receptors (Town et al., 2005).

Commitment to the M1 macrophage lineage (Satoh et al., 2010) is defined by the activation of a member of the interferon-regulatory factor (IRF) family. IRF5 activates genes encoding for inflammatory cytokines, such as TNFs, IL6, IL12 and IL23, and tumor suppressors (Ouyang et al., 2007; Krausgruber et al., 2011). M2 polarization is controlled by IRF4

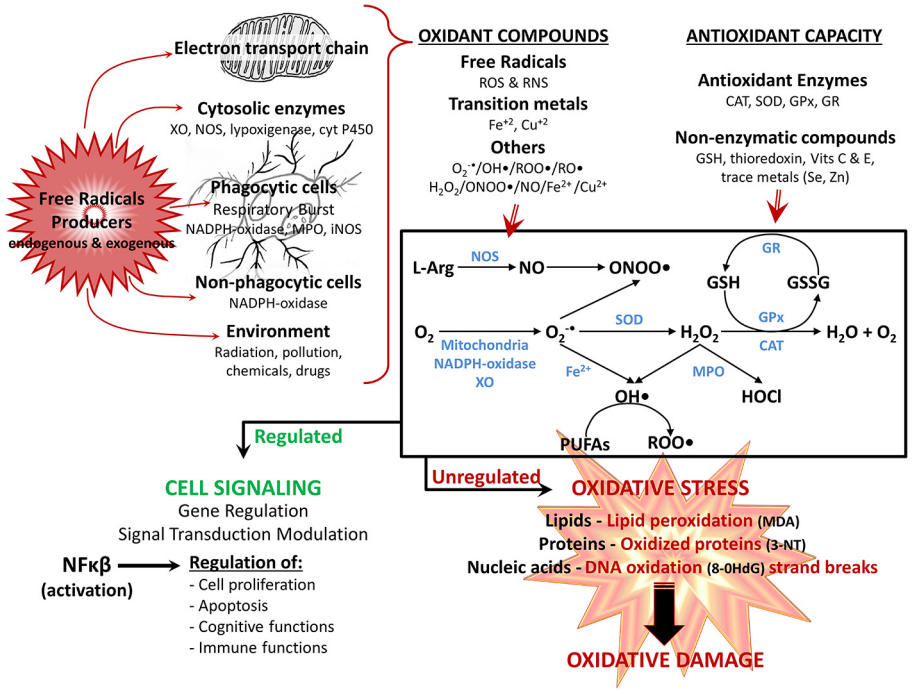

FIGURE 1 | Reactive species participate in normal cellular function or in pathological mechanisms depending on their overproduction. Reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**), are produced through several mechanisms by the cell: the electron transport chain in mitochondria, various cytosolic and membrane enzymes (i.e., xanthine oxidase (**XO**), nitric oxide synthase (**NOS**), NADPH oxidase complex, etc.), as well as exogenously provided by the environment. At the same time, cells have several antioxidant defense mechanisms for detoxifying ROS and RNS, including enzymes (i.e., superoxide dismutase (**SOD**), catalase (**CAT**), glutathione peroxidase (**GPx**), glutathione reductase (**GR**) and nonenzymatic antioxidants (i.e., reduced glutathione (**GSH**), vitamins **E** and **C**. The main generation pathways of ROS and RNS are also shown: the reduction of O_2 occurs by diverse mechanisms (i.e., mitochondria, XO, NADPH-oxidase complex) leading to formation of superoxide anion (**$O_2^{\bullet-}$**); which is easily transformed to hydrogen peroxide (**H_2O_2**) either nonenzymatically or by SOD. H_2O_2 is converted to H_2O by CAT, or by GPx, which together with the GR regenerate GSH. In addition, under stress conditions and high concentration of transition metal (i.e., iron ions—**Fe**), O_2 •- can generate hydroxyl radical (**OH•**), which in turn can react with polyunsaturated fatty acids (**PUFAs**) and generate peroxyl radical (**ROO•**). Finally, O_2 •- can react with nitric oxide (**NO**; depending on NOS), producing the highly reactive peroxinitrite (**ONOO•**) anion, whereas H_2O_2 is converted to hypochlorous acid (**HOCl**) by myeloperoxidase (**MPO**). The balance between oxidants compounds and antioxidant defense determines the end result. Optimal physiologic levels leads to beneficial effects, with ROS and RNS acting as second messengers in intracellular signaling cascades (modulation of gene regulation and signal transduction pathways, mainly by activation of NFκB), regulating several physiological functions (i.e., cognitive and immune functions). However, when overproduction of ROS/RNS is higher than the antioxidant system, the equilibrium status favors oxidant vs. antioxidant reactions, leading to **oxidative stress**, in which ROS/RNS have harmful effects, because of their reaction with various macromolecules (lipids, proteins and nucleic acids), contributing to cellular and tissue oxidative damage, and the development of age-related impairments. **Oxidation products**: 3-NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxy-2-deoxyguanosine; malondialdehyde (**MDA**); alkoxyl radical (**RO•**).

(Satoh et al., 2010; Krausgruber et al., 2011). Cyclic AMP-response element binding protein (CREB)–mediated induction of transcription factor C/EBPβ upregulates M2-specific genes (Ruffell et al., 2009), whereas activation of transcription factor nuclear factor *kappa*-light-chain-enhancer of activated B cells (NFκB)-p50 is associated with the inhibition of M1-activation genes (Porta et al., 2009). Secretion of IL4, IL10 and TGFβ by M2-activated macrophages, promote humoral immune responses and down-regulate M1-mediated responses, inhibiting several inflammatory functions (Town et al., 2005). Originally, it was thought that M2 activation resulted in protective functions. However, there is evidence that M2 cytokines such as IL4, IL5, IL9, and IL13 also result in the induction of some chronic inflammatory processes (Wynn, 2003). As for regulatory macrophages; they appear to arise at later stages of adaptive immune responses, being their primary role limiting inflammatory activation (Mosser, 2003). Regulatory macrophages appear to be generated through several signaling pathways, involving extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK; Lucas et al., 2005; Mosser and Edwards, 2008).

Microglia are activated in nearly all CNS diseases (Kreutzberg, 1996; Hanisch and Kettenmann, 2007; Neumann et al., 2009), producing and secreting a broad spectrum of inflammatory mediators, such as eicosanoids, cytokines (Nakamura, 2002; Kim and de Vellis, 2005; Tichauer et al., 2007), chemokines, ROS, nitric oxide (NO·), small metabolites, proteases (ea. α-antichymotrypsin and α-antitrypsin), and inflammatory markers (ea. serum amyloid P and C-reactive protein; Li et al., 2007; Tichauer et al., 2007; Neumann et al., 2009; Lue et al., 2010).

Those inflammatory mediators regulate innate immune defense and have profound effects on neuronal properties, modifying synaptic function (Selkoe, 2002; Di Filippo et al., 2008). In addition, microglia can also induce bystander damage of neurons, especially under conditions of strong or long lasting stimulation, and depending on the environmental context (Li et al., 2007; von Bernhardi, 2007). In fact, cytotoxic activation of microglia is associated with neuronal loss and decline of cognitive and neurobehavioral function (Cagnin et al., 2001; Kim and de Vellis, 2005; Block et al., 2007). Nevertheless, microglia also secretes trophic factors and modulator cytokines, being active partners in neuroprotection.

Neuroinflammation establishes a complex interaction with oxidizing agents through redox sensors present in enzymes, receptors, and transcription factors. Those factors affect neuron-glia crosstalk and neuronal function (Liu et al., 2012), resulting later in neurodegenerative changes (Raj et al., 2014). Signal transduction of various cytokines, themselves critical mediators of oxidative stress, neuroinflammation, and even neurodegenerative changes, are modified by the redox status (Mrak and Griffin, 2005; Kierdorf et al., 2010). Oxidative stress, a result of the equilibrium between production and detoxification of radical species (**Figure 1**), further increases inflammatory cytokines, creating a vicious cycle (Rosales-Corral et al., 2010), and affects the maintenance of cellular homeostasis and cell survival (Satoh and Lipton, 2007).

Mitochondria were often thought to be the main responsible for ROS overproduction and oxidative stress. However, NADPH oxidase (NOX) enzymes participation is also an important ROS-generating system (Bordt and Polster, 2014). Activation of the phagocyte NADPH oxidase (NOX2) in microglia, plays a role in neuroinflammation, but appears also to contribute to neuronal death under pathologic conditions (Qin et al., 2013; Jiang et al., 2015). Moreover, ROS production can also depend on other NOX isoforms, which are detected also in astrocytes and neurons (Nayernia et al., 2014). Whereas ROS derived from normal NADPH oxidase function is required for processes such as neuronal signaling, memory, and central homeostasis (Jiang et al., 2015), overproduction of ROS contributes to excessive oxidative stress, resulting in neuronal dysfunction and neurotoxicity (Zhang et al., 2014). ROS regulates several signal transduction pathways, including for some trophic factors and hormones. NFκB is a transcription factor activated by ROS and inflammatory mediators that participates both in protective and deleterious responses, depending on the context of stimulation that will result in the co-activation of various signaling pathways. It activates genes regulating cellular survival, growth, differentiation, inflammation, and cell death. Under non-stimulated conditions, NFκB is kept inactive by IκB (inhibitor of κB) in the cytoplasmic compartment. High concentrations of ROS inactivate NFκB through oxidation of its p50 subunit, inhibiting its DNA binding. In contrast to the inhibitory effect of high ROS levels, moderate levels of ROS lead to the sequential phosphorylation, polyubiquitination and degradation of IκB, allowing the activation of NFκB (**Figures 1**, **2**). Once activated, and depending on the context, NFκB plays a pro-survival role by inhibiting c-Jun N-terminal kinases/stress-activated protein kinase (JNK) and caspase cell death pathways and upregulating transcriptional activation of anti-apoptotic proteins and genes involved in decreasing mitochondrial ROS (mtROS), especially those coding for manganese superoxide dismutase (MnSOD; Patten et al., 2010). TNFα also activates NFκB associated with neuroprotection against β-amyloid (Aβ) neurotoxicity *in vitro* (Barger et al., 1995), and NFκB activates anti-apoptotic responses and protects neurons from excitotoxicity and ischemic brain injury (Pennypacker et al., 2001; Bhakar et al., 2002; Mattson, 2005).

On the other hand, NFκB activation can also be detrimental. NFκB has a key role in the initiation and amplification of inflammation through its response to inflammatory stimuli mediated by TNFα or IL1, leading to the induction of several cytokines and chemokines. Activation of NFκB and MAPK pathways are conspicuous in oxidative stress- (Chen et al., 2009; Chongthammakun et al., 2009) and Aβ-induced (Song et al., 2004) neuronal cell death. In addition to NFκB, other transcription factors are activated by inflammatory conditions, such as peroxisome proliferator-activated receptor gamma (PPARγ) and signal transducer and activator of transcription (STAT-1) and have also been implicated in Alzheimer's disease (AD; Sastre et al., 2006; Cho et al., 2007).

The brain is particularly vulnerable to oxidative stress. Vulnerability depends on its: (i) high oxygen metabolic rate (consumes approximately 20% of the total consumption of oxygen of a mammal), (ii) high dependence on oxidative metabolism for obtaining energy, (iii) high content of iron, an endogenous catalyzer for the generation of ROS and reactive nitrogen species (RNS), (iv) lower content of antioxidant enzymes compared with other organs (Floyd and Hensley, 2002; Mattson et al., 2002); and (v) low ability to eliminate mutations not removed by cell replacement as consequence of the post-mitotic nature of neurons. Aged, or injured brains of any sort, show oxidative modifications in nucleic acids, proteins, lipids, and sugars (**Figure 1**). Several of those oxidative damage and changes result in a loss of function (Lovell et al., 2001; Halliwell, 2006).

Age-Related Changes of Microglia

Microglial cell changes have been documented in aging. However, many of those changes are also observed in neurodegenerative conditions. Thus, it is still unclear whether these changes are reactive to the underlying pathophysiology. Although there is an agreement on the fact that degenerative diseases are not the natural continuous progression of age-related decline, both aging and neurodegenerative disease appear to be highly multifactorial conditions that also share many relevant factors. Aging is a mayor risk factor for the development of many neurodegenerative diseases. Furthermore, neuroinflammation and oxidative stress (both reportedly associated with non-pathological aging in humans and animal models) are common features for several disease phenotypes. Studies in cell cultures and animal models suggest the existence of altered activation states and cellular senescence in the aged brain. Not only aging

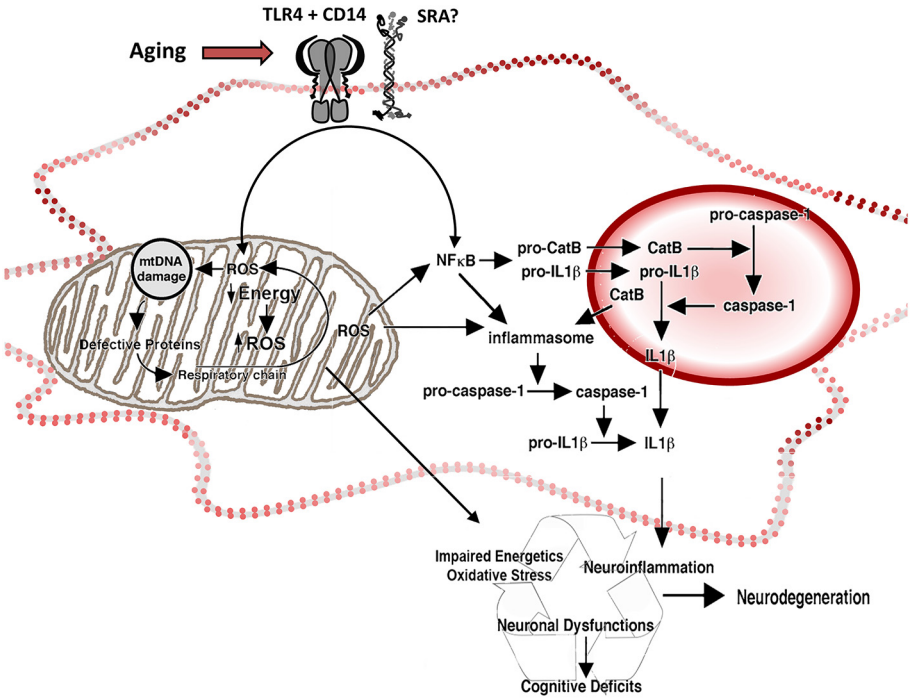

FIGURE 2 | Reactive oxygen species and inflammation in the aged microglia. Representation of the participation of mitochondria and lysosomes in the increased production of ROS and inflammatory cytokines by aged microglia. Increased intracellular ROS activate redox-sensitive NFκB through a pathway mediated by mitochondrial ROS (associated with decreased energetic production and increased release of ROS by the electron transport chain) and a ROS-independent pathway, potentiating neuroinflammation. The activation of NFκB induces production of pro-CatB and pro-IL1β, and the activation of inflammasome in the cytoplasm. Pro-CatB is processed into CatB in the lysosome, which in turn, mediates the activation of pro-caspase-1 to caspase 1 and increases the processing of pro-IL1β, releasing increased amounts of IL1β both in the phagolysosome and the cytoplasm, as well as potentially potentiate apoptosis. Changes on the expression of pattern recognition receptors, like TLR4 CD14 and SRA, result in changes on neuroinflammatory activation and oxidative stress by activating NFκB and the release of ROS.

appears to be a key risk factor for neurodegenerative as well as other chronic diseases (Mosher and Wyss-Coray, 2014; Cho et al., 2015), but the presence of those diseases potentiate also the appearance of aging and senescence related markers (Baron et al., 2014; Mosher and Wyss-Coray, 2014; Bachstetter et al., 2015).

There is high heterogeneity of microglia in various neurodegenerative diseases and those phenotypes share common characteristics with aging (Bachstetter et al., 2015) as well as the pattern of microglia gene expression is shared by aging and neurodegenerative conditions (Holtman et al., 2015). Moreover, many of the changes described in aged microglia represent changes that occur during aging; meaning that, they do not appear when reaching a certain age threshold, but they change through life, as the individual ages. Analysis of transcriptome data from postmortem studies of frontal cortex from 381 healthy individuals with ages spanning from young teenagers to people older than 80 years of age, show that microglia gene markers assemble into a transcriptional module in a gene co-expression network (Wehrspaun et al., 2015), whose expression pattern show a negative correlation with age. Genes that encode microglia surface receptors for neuron and/or microglia crosstalk are especially affected. In addition, they found that microglia are controlled by brain-expressed transcription factors, including *RUNX1*, *IRF8*, *PU.1*, and *TAL1* (Kierdorf and Prinz, 2013), which are master regulators for the age-dependent microglia module. As the authors highlighted, identification of age-dependent gene modules in adulthood are relevant for understanding critical periods for susceptibility to late-onset diseases (Wehrspaun et al., 2015).

Senescent microglia display morphological changes (**Figure 3**), with fewer and shorter processes, increased soma volume, and formation of spheroid swellings, which is referred as "dystrophic microglia" (Streit et al., 2004b; Conde and Streit, 2006a,b; Streit, 2006; Flanary et al., 2007). Microglia co-localize with neurodegenerating neurons, and show clumping, with loss of their homogeneous tissue distribution, and accumulation of phagocytic inclusions (Hart et al., 2012; Tremblay et al., 2012; Hefendehl et al., 2014). Live imaging shows that the dynamic response of microglia to injury changes with age. Young microglia increase their motility and extend ramifications rapidly when exposed to ATP, an injury-associated signal, or to a focal tissue injury. In contrast, aged microglia are less dynamic and ramified and further reduce their dynamism when exposed to ATP. On the other hand, disaggregation of aged microglia from the site of injury becomes slow, indicating

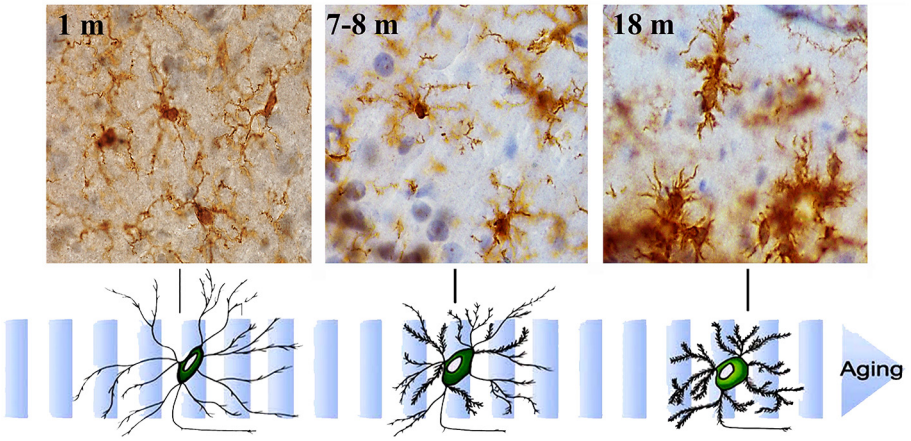

FIGURE 3 | Aging-related morphological changes of microglia. Microglial cell morphology changes with aging. Immunohistochemistry for Iba-1 (a constitutive identity marker for monocyte-macrophage cells) and counterstaining with hematoxylin of hippocampal sections from animals of different ages (1- to 18-month old). Microglia obtained from young mice have a small cell body and very long and slender ramifications. As mice age, microglia gradually show bigger cell bodies and progressively shorter and thicker cell processes.

that aged microglia tend to show sustained responses (Damani et al., 2011). Both in aging (Flanary and Streit, 2004) and in AD (Flanary et al., 2007), microglia show telomere shortening and decreased telomerase activity, which are speculated to be one of the factors underlying the diminution of some functional activities, such as clearance (phagocytosis plus effective removal of the compounds) and basal proliferation (Harry, 2013). Reduced microglia replication could also result in a depletion of healthy microglia, favoring the participation of more senescent and dysfunctional cells (Mosher and Wyss-Coray, 2014).

Activated microglia are the primary cellular source of both inflammatory molecules and oxidative products (**Figure 4**). (Pawate et al., 2004; Qin et al., 2005b; Hayashi et al., 2008). Microglia from aged brains show increased basal production of IL6 and enhanced lypopolysaccharide (LPS)-induced IL6 and IL1β, compared with microglia from young mice brains in culture (Ye and Johnson, 1999; Sierra et al., 2007). They appear to be activated also under normal physiological conditions. In aging, mild stimulatory events or minor injuries, otherwise easily solved, could induce damage and initiate a disease process. TGFβ1 is a strong regulator of neuroinflammation and cytotoxicity and its signaling pathway could be part of the switch mechanism from protective to deleterious activation of microglia. Its downstream canonical signaling involves the Smad pathway, which transduce extracellular signals from ligands acting as transcription factors (Derynck and Zhang, 2003), as well as a complex Smad independent signaling (Weiss and Attisano, 2013). TGFβ1 secreted by hippocampal neurons and astrocytes regulates microglial cell activation, attenuating the release of inflammatory cytokines and reactive species (Chen et al., 2002; Mittaud et al., 2002; Herrera-Molina and von Bernhardi, 2005; Herrera-Molina et al., 2012), protecting neuronal cells *in vitro* (Hu et al., 1995; Lieb et al., 2003; Herrera-Molina and von Bernhardi, 2005) and promoting microglia-mediated Aβ phagocytosis and degradation (Wyss-Coray et al., 2001). These regulatory effects of TGFβ1 are mediated by Smad3-dependent mechanisms (Flores and von Bernhardi, 2012; Tichauer and von Bernhardi, 2012), as well as the reported inhibition of lipopolysaccharide (LPS)-induced macrophage and microglial activation (Werner et al., 2000; Le et al., 2004). TGFβ1 Smad3 pathway also participates in the inhibition of the production of radical species induced by inflammatory stimuli and in the induction of amyloid-β (Aβ) phagocytosis *in vitro* (Tichauer and von Bernhardi, 2012).

TGFβ1 levels are elevated in aged individual (Blobe et al., 2000; Tichauer et al., 2014). However, recent reports show that induction of the Smad3 pathway by inflammatory conditions is decreased in normal aging (Tichauer et al., 2014). Interestingly, this signaling pathway is impaired in AD patients and mouse models for AD, resulting in Aβ accumulation, Aβ-induced neurodegeneration, and neurofibrillary tangle formation (Tesseur et al., 2006; Ueberham et al., 2006). Evidence gathered over the last two decades indicate that TGFβ signaling impairment often lead to neuroinflammation, neuronal dysfunction and neurodegenerative changes, and could be involved in the pathogenesis of neurodegenerative diseases (Tesseur and Wyss-Coray, 2006). Given the complex signaling pathway activated by TGFβ, which in addition to the Smad pathway also activates Smad-independent signaling, including ERK/MAPK, P38 MAPK, JNK, and PI3K (Derynck and Zhang, 2003; Weiss and Attisano, 2013), a decreased activation of Smad3 in an environment presenting elevated levels of TGFβ, as observed in aging, could result in an increased activation of MAPKs and PI3K, which are signaling pathways also involved in inflammatory activation. Such an imbalance on the signaling activated by TGFβ could explain, at least partially, the maintenance of increased levels of microglial cell activation, oxidative stress and mild neuroinflammation, although TGFβ1,

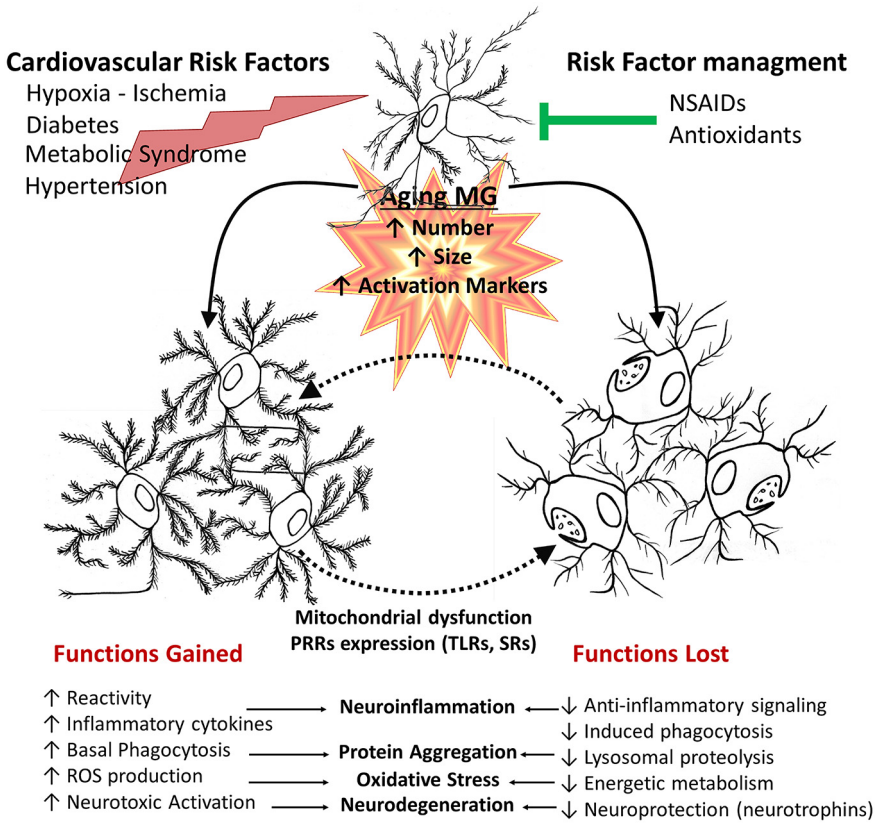

FIGURE 4 | Age-related changes of microglial cell function. In aged brains, there is an increased number, size and activation of microglia. This is affected by additional systemic pathophysiological changes associated with other age related changes, environmental factors and disease processes, such as cardiovascular risk factors and metabolic syndrome or injuries. Deleterious processes further promote an inflammatory environment, increasing cytotoxic microglial cell activation, whereas risk factor management and pharmacological interventions can promote a healthy aging. Aged microglia changes depend both on gained and lost functions. They have increased basal phagocytic activity, although a reduced capacity to induce phagocytosis when stimulated, together with reduced lysosomal activity, resulting in a decreased clearance activity. Microglia also shows an increased production of inflammatory cytokines and reactive species. Those changes result in a shift of balance towards decreased protective functions and an increased neurotoxicity.

one of the main regulatory cytokines decreasing inflammatory activation, is increased in aged mice (Tichauer et al., 2014). Those results indicate that TGFβ1-Smad3 signaling could be a therapeutic target for AD treatment.

Another alternative is that stimuli that normally would trigger a protective response, in conditions of age-related impairment of normal homeostatic mechanisms result in a persistent activation, which is associated, for example, to a robust induction of oxidative stress (**Figures 4**, **5**; von Bernhardi, 2007; Herrup, 2010), or to the upregulation of NFκB. In fact, NFκB response is age-dependent, and it is another candidate for age-dependent changes due to its role in the regulation of immunity, inflammation, and cell death (Adler et al., 2007). Blockade of NFκB in aged mice has been reported to reverse the gene expression program and cell morphology, "rejuvenating" old mice (Adler et al., 2008). TNFα signaling involves NFκB, resulting in a beneficial or detrimental response depending on the age and the type of stimuli. Stimulation of 24 month-old rat neurons with TNFα plus Aβ is toxic, whereas those same stimuli are protective for 10 month-old neurons (Patel and Brewer, 2008). The down-regulation of TNFs receptors TNFR1 and TNFR2 signaling observed in aging results in defective NFκB activation and fails to provide a neuroprotective response against Aβ toxicity by TNFα (Patel and Brewer, 2008). NFκB accumulates in the nuclei of old neurons; an effect that is also produced by blocking TNFR2. An alternative explanation for the failure of NFκB to activate protective pathways could depend on high concentrations of ROS (Parihar and Brewer, 2007), and the oxidized redox state of aged cells (Parihar et al., 2008). The redox state of NFκB could be a control mechanism regulating its availability (Sulciner et al., 1996). It is unclear whether the over-production of ROS, through a vicious cycle in the aging mitochondria, may activate redox-sensitive NFkB, thereby provoking excessive inflammation in the aged brain (Hayashi et al., 2008; Nakanishi and Wu, 2009; **Figure 2**).

When exposed to endotoxins like LPS, microglia derived from adult mice secrete high amounts of ROS, whereas young animals microglia predominately produce NO·, with little ROS (Tichauer et al., 2014). Aged microglia become more inflammatory

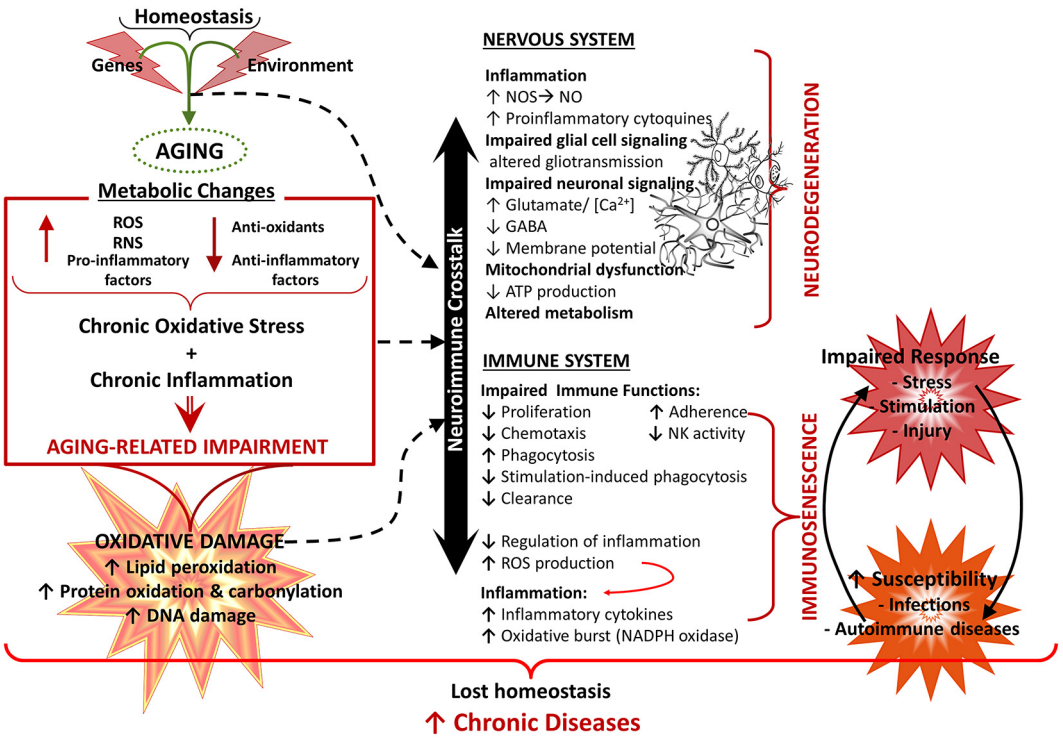

FIGURE 5 | Aging of the nervous and immune system and the neuroimmune crosstalk. Healthy aging of the nervous and immune systems depend both on genetic and environmental (lifestyle) factors. Aging is associated with a state of low grade chronic oxidative stress and inflammation (with production of reactive mediators and inflammatory compounds and a decreased antioxidant and anti-inflammatory capacity), which appear to be the cause of an important part of age-related deterioration of the nervous and the immune systems, as well as of the neuroimmune communication. Because of their complex functions, the central nervous system (CNS) and the immune system are especially vulnerable to oxidative damage (i.e., lipid peroxidation, protein oxidation, DNA damage), which contributes to oxidative stress and inflammation. Age-related changes in the immune function, known as immunosenescence, results in increased susceptibility to infections and cancer, inflammation and autoimmune diseases. In the CNS, oxidative stress has a negative impact on function, leading to mitochondrial dysfunction and impaired energetic metabolism, altered neuronal and glial signaling. There may be disruption of the cycle glutamate-glutamine and increased levels of neuronal calcium, which are involved in mechanisms of neuronal damage leading to loss of function, excitotoxicity and apoptosis. In addition, dysfunction of the neuron-glia crosstalk leads to a chronic neuroinflammation, which promotes a prolonged activation of microglia and further induction of dysfunction and degenerative changes. All these alterations contribute to functional decline and the development of neurodegenerative diseases. **NO**, nitric oxide; **NOS**, nitric oxide synthase; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species.

than their younger counterparts upon systemic inflammatory stimulation; thus exacerbating neurodegenerative changes (Combrinck et al., 2002; Cunningham et al., 2005; Godbout et al., 2005; Sierra et al., 2007). Systemic inflammation also causes aged microglia to become more responsive than young microglia, increasing production of inflammatory cytokines (IL1β, IL6 and TNFα). The resulting exacerbated response to inflammatory challenges appears to depend on the priming of microglia by previous activation experience. Primed microglia undergoes a phenotypic shift towards a sensitized state, responding to a secondary "triggering" stimulus more rapidly and robustly than non-primed cells (Harry, 2013). Therefore, the exacerbated response to stimuli of aged microglia can contribute to neuronal damage (**Figure 5**) and the onset of chronic diseases (Perry et al., 2003, 2007; Perry, 2004).

Age-related changes on cell response involve changes on microglia receptors (**Figures 2**, **4**). Aged microglia show upregulation of Toll-like receptors (TLRs), and TLR4 co-receptor CD14 (Letiembre et al., 2007), as well as age-related changes in signal transduction of TLR4. There are changes in the expression profile of scavenger receptors (SRs; Yamamoto et al., 2002; Hickman et al., 2008). TLRs, CD14, and SRs are pattern recognition receptors (PRRs), key participants of the host defense response and the phagocytosis of pathogen-associated molecules pattern (PAMPs) and damage-associated molecules pattern (DAMPs), being crucial for the innate immune response The activation of these receptors by diverse ligands is associated with activation of microglial cell (Godoy et al., 2012; Murgas et al., 2012, 2014), production of inflammatory mediators, and uptake of pathogens and macromolecules, including Aβ (Alarcón et al., 2005). Thus, changes on their expression pattern affect cell activation (Cornejo and Von Bernhardi, 2013). In addition, aged microglia also express some surface antigens that are not normally expressed by their young counterparts, including the major histocompatibility complex II (MHCII), associated with antigen presentation, and ED1, the rodent equivalent of CD68, associated with phagocytosis. Regardless of the increased CD68, aged microglia are not better

phagocytes than young microglia (Floden and Combs, 2011). In fact, aged microglia appear to have a decreased ability to phagocytose Aβ compared with microglia from young mice (Floden and Combs, 2011). We observed that although basal phagocytosis by microglia obtained from 1-year old mice is slightly increased compared with young mice, phagocytosis fails to be induced by TGFβ (Tichauer et al., 2014) or LPS (Cornejo et al., 2014), and is not coupled to an effective clearance machinery (**Figure 5**). Moreover, in addition to phagocytosis, protein homeostasis is impaired at several levels, including chaperone-mediated protein folding and stability, protein trafficking, protein degradation and autophagy. A major consequence of these impairments is the aggregation of abnormal proteins, which is an important neuropathological finding in several neurodegenerative diseases, such as Parkinson's disease (PD) and AD (Taylor and Dillin, 2011). Taken together, age-related changes in receptors expression could account for alterations observed in microglial cell function, providing insight on cell phenotypes that could play a role in the pathophysiological changes leading to neurodegenerative diseases.

Autophagy capacity can regulate mitochondrial integrity, ROS production, and subsequent NLR family, pyrin containing 3 (NLRP3) inflammasome activation (Nakahira et al., 2011; Zhou et al., 2011; Salminen et al., 2012). NLRP3 activation is negatively regulated by autophagy, because damaged mitochondria producing high amounts of ROS are removed by autophagy. In fact, inhibition of autophagy triggers accumulation of damaged mitochondria (Zhou et al., 2011), which produce more ROS.

Mitochondrial DNA (mtDNA), which encodes components of the mitochondria electron transfer complexes, is highly susceptible to ROS-mediated damage, due to its close proximity to the ROS generated by the respiratory chain and to its decreased number of protective histones and DNA-binding proteins. Aging-related accumulation of mtDNA damage results in a reduced expression of mitochondria electron transfer complexes, in especial complexes I and IV, because they contain a relatively large number of mtDNA-encoded subunits. The reduced activity of complex I further facilitates the generation of ROS (Lin et al., 2002), establishing a vicious cycle (Kang et al., 2007; **Figure 2**). Most cells have protective mechanisms, depending on enzymatic breakdown or scavenging of ROS (**Figure 1**). However, antioxidant systems appear to be less functional in the brain, which can lead to persistent increased levels of ROS and RNS reacting with the various target molecules (Halliwell, 2006).

Functional decline of lysosomes and mitochondria in microglia produces an exacerbated generation of ROS and inflammatory mediators, which could further promote microglia aging (Hayashi et al., 2008). Accumulation of mitochondrial DNA oxidative damage in microglia during aging, increases ROS production. The increased intracellular ROS, in turn, activates the redox-sensitive nuclear factor *kappa* B, inducing neuroinflammation (Nakanishi and Wu, 2009), which in turn also promotes oxidative stress. Mitochondria-derived ROS and cathepsin B, are also involved in the microglial production of interleukin-1β (**Figure 2**).

During aging, autophagy efficiency declines and becomes dysfunctional, resulting in the accumulation of waste materials within cells (Salminen et al., 2012). On the other hand, induction of phagocytosis on LPS-primed microglia can cause lysosomal damage. The release of cathepsin B (CatB), a lysosomal cysteine protease, into the cytoplasm triggers the activation of the NLRP3, leading to the production and secretion of IL1β (**Figure 2**) and IL18 (Halle et al., 2008; Hornung et al., 2008). Interestingly, a NLRP3-deficient AD mice model show improvement of their spatial memory deficits, a reduced expression of brain caspase-1 and IL1β, and enhanced Aβ clearance (Heneka et al., 2013). In addition of Aβ, cholesterol crystals is also a major causative factor of age-related diseases such as atherosclerosis, and also shows activation of the inflammasome in a CatB-dependent manner (Duewell et al., 2010; Masters et al., 2010).

Aged Microglia-Related Neuronal Impairment and Neurodegenerative Diseases

Age-dependent changes gradually have a toll on brain homeostasis and function (Herrup, 2010; von Bernhardi et al., 2010), changing glial cell reactivity (von Bernhardi, 2007). Cytotoxic activation of microglia, increased production of inflammatory cytokines, and ROS combined with impaired ability to regulate increased oxidative stress in the aging brain (Conde and Streit, 2006b; von Bernhardi et al., 2010). Those changes appear to be causative factors for neurodegenerative processes, (**Figure 5**; Block et al., 2007) and the associated decline in motor and cognitive functions (Forster et al., 1996; Navarro et al., 2002).

Chronic inflammation induces deficits in long-term potentiation (LTP), the major neuronal substrate for learning and memory, in middle-aged but not in young rats (Liu et al., 2012). Similarly, *in vivo* microinjection of fibrillary Aβ in the cortex of aged rhesus monkeys showed neurodegeneration, tau phosphorylation, and microglial cell proliferation, but not in young monkeys, suggesting that Aβ neurotoxicity is a pathological response of the aging brain (Geula et al., 1998). In this context, microglia upregulated production of IL1β, is possibly implicated in age-associated cognitive impairments (Rachal Pugh et al., 2001; Maher et al., 2006). As mentioned above, aged microglia actively participate in the genesis of neuronal damage in neurodegenerative diseases, through production of inflammatory mediators and ROS (Block et al., 2007), but also because of the impairment of their neuroprotective functions (**Figure 5**). Thus, microglia contribute to the death of dopaminergic neurons in PD, forebrain neurons in AD, and motor neurons in amyotrophic lateral sclerosis (ALS; Boillée et al., 2006; Mount et al., 2007). Similarly, TNF*α* promotes PD progression (McCoy et al., 2006), whereas the absence of TNFR1 protects against AD- and PD-like disease in mice (Sriram et al., 2002; He et al., 2007).

Neurodegenerative diseases often have increased generation of RNS and ROS as an early event (Perry et al., 2002; Shi and Gibson, 2007), which can contribute to neuronal cell

injury via various redox reactions (**Figure 1**). Deficiency in antioxidant enzymes, such as superoxide dismutase (SOD), increases disease associated phenomena (Li et al., 2004a), increasing tau phosphorylation (Melov et al., 2007), and amyloid and tau aggregation (Li et al., 2004a), and accelerates behavioral impairment (Esposito et al., 2006). Thus, oxidative damage in the brain of AD patients and animal models is more abundant than that observed in age-matched control individuals. Conversely, increased expression of antioxidant enzymes attenuates AD phenotype (Dumont et al., 2009).

There are additional mechanisms for reactive species-related impairment, NO· target cysteine residues of proteins to form S-nitrosothiols (SNOs). The interaction with proteins that are targets of S-nitrosylation represents NO· signal transduction (Hess et al., 2005). S-nitrosylation switches the on-off functions of receptors, GTPases, and transcription factors, and can affect mitochondrial function. NO· reversibly inhibits complexes I and IV (Clementi et al., 1998), further increasing release of ROS by mitochondria, further promoting dysfunction of mitochondrial dynamics (Bossy-Wetzel and Lipton, 2003; Barsoum et al., 2006). Moreover, S-nitrosylation modulates GTPase activity of the mitochondrial fission protein dynamin-related protein 1 (Drp1), favoring altered mitochondrial dynamics, synaptic damage, and eventually neuronal death (Cho et al., 2009). Other examples relevant for aging and neurodegeneration are: (i) the S-nitrosylation of protein-disulfide isomerase (PDI, an enzyme relevant for the maturation and transport of unfolded secretory proteins), which abolishes PDI-mediated inhibition of neurodegenerative changes triggered by endoplasmic reticulum (ER) stress, misfolded proteins, or proteasome inhibition (Uehara et al., 2006); and (ii) the S-nitrosylation of ApoE, resulting in changes of its interaction with low-density lipoprotein (LDL) receptors (Abrams et al., 2011).

Microglia and Alzheimer's Disease

Neurodegenerative diseases, including AD, involve several converging disease mechanisms, generating a functional interplay between neurons and glial cells (**Figure 5**). The AD brain is characterized by the presence of senile plaques, constituted by aggregated Aβ, and neurofibrillary tangles (NFTs), formed by hyper-phosphorylated tau, as well by synapse and neuronal loss (Uylings and de Brabander, 2002), and glial cell activation (Kim and de Vellis, 2005; Jellinger, 2006; Heneka and O'banion, 2007; von Bernhardi, 2007; von Bernhardi et al., 2010). Interestingly, Alzheimer, on his original descriptions, already stated that these lesions were markers of an upstream process rather than the disease cause (Davis and Chisholm, 1999). The fact that brain innate immune response could be involved in the genesis of neurodegenerative diseases (Nguyen et al., 2002; Björkqvist et al., 2009; von Bernhardi et al., 2010), lead to re-consider the role of Aβ and propose glia to be a leading factor in the pathology of AD (von Bernhardi, 2007). The hippocampus, one of the regions affected early by neurodegeneration in AD, is one of the most densely populated by microglia together with the *Substantia nigra*. However, most scientists who adhere to the "amyloid cascade hypothesis" of AD, view Aβ as the cause of AD and neuroinflammation just as a consequence of glia activation (Akiyama et al., 2000; Heneka and O'banion, 2007; Hirsch and Hunot, 2009).

Microglia are intimately associated with Aβ plaques in AD, but not with the diffuse Aβ plaques of the normal aged brain (Itagaki et al., 1989; von Bernhardi et al., 2001; von Bernhardi, 2007; Hashioka et al., 2008; Heurtaux et al., 2010). The trigger for microglia activation is unclear, but the invasion of plaques by active microglia has been reported in AD transgenic mice models, when Aβ is injected into the brain or in *in vitro* experiments (von Bernhardi et al., 2001; Alarcón et al., 2005; Reed-Geaghan et al., 2009; Njie et al., 2012; Thanopoulou et al., 2010). Their activation by Aβ (Simard et al., 2006; Hashioka et al., 2008; Koenigsknecht-Talboo et al., 2008) results in cell transformation (Husemann et al., 2001). Microglia aging is associated with several mechanisms underlying the formation and accumulation of Aβ aggregates. Microglia clearance (phagocytosis plus degradation) of Aβ is reduced leading to its initial accumulation (Floden and Combs, 2011; Zhao et al., 2014), as well as its capacity to migrate (Sheng et al., 1998; Damani et al., 2011) and shift among inflammatory activation patterns towards a more phagocytic stage (Sierra et al., 2007; Streit et al., 2009; Schuitemaker et al., 2012). Similar results have been reported on AD patients (Mawuenyega et al., 2010). There is an age-related impairment of phagocytosis (Harry et al., 2000; Zhao et al., 2014) and clearance. Clearance by both microglia and astrocytes appears to depend on peroxisome proliferator-activated receptor-γ (PPARγ) and apolipoprotein E (apoE) levels, which promote the proteolytic clearance of soluble forms of Aβ (Mandrekar-Colucci et al., 2012). In addition, human genetic studies indicate that coding variants of TREM2, a regulator of microglia activation and phagocytosis, are suggestive of microglia immune senescence (Guerreiro et al., 2013), and results in a substantial risk for AD. Plaque-associated reactive microglia in these animals show enhanced staining for TNF*α* and IL-1β (Benzing et al., 1999). Neuroinflammation as well as other stressors promote production and release of Aβ (Lee et al., 2007; Mosher and Wyss-Coray, 2014) as well as its amyloidogenicity, favoring its aggregation. However, acute increased levels of various inflammatory factors, including IL1β and IL6 are associated with activation of glial cells and reduced amyloid pathology (Chakrabarty et al., 2010; Jiang et al., 2015), although chronic neuroinflammation fails promoting amyloid removal. Promotion of Aβ production and aggregation has been also observed secondary to microglia-related ROS through a stress response or depending on oxidative modifications of the peptide (Giasson et al., 2002).

Importantly, Aβ is also clearly indicated as a source of oxidative stress (Varadarajan et al., 2000), as Aβ activates microglia to produce extracellular superoxide radical (O_2·-; Qin et al., 2002; Bamberger et al., 2003), and can be a potent inducer of NFκB via the induction of intracellular ROS (Lee et al., 2005; Valerio et al., 2006) as well as through the TNFR1 signaling,

which results in neuronal apoptosis (Li et al., 2004b; Valerio et al., 2006).

In addition to the role of oxidative stress in neuron dysfunction and degeneration, secondary to Aβ neurotoxicity, excitotoxicity, aggregation of proteins, and impaired calcium metabolism (Kuchibhotla et al., 2008; Lopez et al., 2008; Santos et al., 2010a,b), ROS appears to be a common mediator unifying the spectrum of cellular mechanisms leading to AD (**Figure 6**). Oxidative damage of the brain of AD patients and animal models include lipid peroxidation (Praticò et al., 1998; Butterfield and Lauderback, 2002; Butterfield, 2002; Butterfield et al., 2002), and oxidation of proteins and nucleic acids (Nunomura et al., 2001, 2004). RNA and DNA oxidation could impair protein synthesis, DNA repair, and transcription, and could eventually lead to cell death (**Figure 1**; Ding et al., 2006). Oxidation of mtDNA is 10-fold more abundant than that of nDNA. Increased mtDNA oxidation could lead to the reported mitochondrial abnormalities, which may contribute to the increase of O2·- leakage, ultimately resulting into elevated oxidative stress (Swerdlow, 2007; Swerdlow et al., 2010).

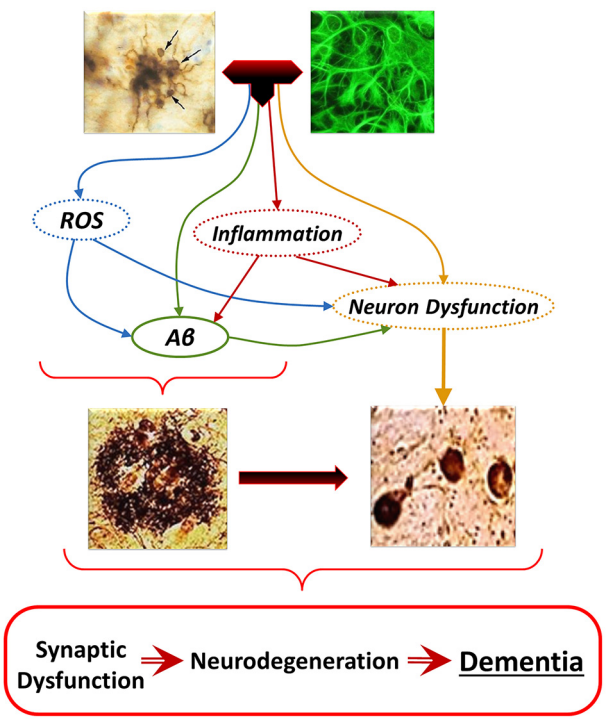

FIGURE 6 | The "Glial Cell Dysregulation Hypothesis" of Alzheimer's disease (AD). The glial cell dysregulation hypothesis proposes that AD has its cause on changes on the activation of microglia and on impaired regulation, which become increasingly cytotoxic decreasing their defensive functions. Impaired activation results in oxidative stress, persistent neuroinflammation and neuronal dysfunction, all of which can also induce production and aggregation of Aβ, and additional neuronal dysfunction. Inflammatory activation, secondary to aging and to various forms of stimuli or injury through life, can result in glial cell dysregulation. Dysregulated activation of glia, through the abnormal release of cytokines, reactive species, and other mediators, contributes to the increased expression of Aβ as well to functional and degenerative changes of neurons, perpetuating abnormal activation of glia, synaptic dysfunction and cell damage.

Glia actively promote neuronal dysfunction and neurodegeneration (von Bernhardi, 2007) through oxidative stress mechanisms by: (i) modifying intracellular proteins and lipids (Lovell et al., 2001; Halliwell, 2006; Zhu et al., 2007); (ii) inducing mitochondrial dysfunction, which increases production of ROS, and activates caspases, activating cell death pathway (Baloyannis, 2006; Lin and Beal, 2006a,b) and ATP depletion (Baloyannis, 2006); (iii) facilitating formation of ubiquitinated aggregates of misfolded proteins (Oddo, 2008) as consequence of the impairment of energy-dependent ubiquitin–proteasome pathway and abnormal phosphorylation of cytoskeleton components (Arnaud et al., 2006); (iv) inhibiting glial cell excitatory amino-acid transporter 2 (EAAT2) activity (Tian et al., 2010) inducing release of glutamate by astrocytes (Lauderback et al., 2001). Overactive glutamate receptors increase intracellular free calcium, causing mitochondrial toxicity (Mahad et al., 2008; Kawamata and Manfredi, 2010) and affect several calcium-dependent enzymes leading to dysfunction and initiation of apoptosis (Mattson and Chan, 2003); and (v) activating microglia (**Figure 4**) and astrocytes to produce and release inflammatory cytokines (von Bernhardi, 2007; Agostinho et al., 2010; Lee et al., 2010; von Bernhardi et al., 2010) and other reactive mediators (NO·, ROS; Zhu et al., 2007; von Bernhardi, 2007; Block, 2008; Agostinho et al., 2010; von Bernhardi et al., 2010). These factors activate signaling pathways of cytokines as well as of eicosanoids produced by cyclooxygenase-2 (COX-2; Wang et al., 2004; Trepanier and Milgram, 2010). Aging and AD also present changes in enzymes involved in glutathione (GSH) metabolism (**Figure 1**; comprehensive view on glutathione peroxidase (GPx), in Toppo et al. (2009). glutathione S-transferase (GST) activity is decreased in the AD amygdale, hippocampus, parietal lobe, and nucleus basalis of Meynert (Lovell et al., 1998). Decreased glutathione S-transferase omega-1 (GSTO1; Li et al., 2003), can be involved in the activation of IL1β (Laliberte et al., 2003), a fundamental component in the early inflammatory response of AD (Grimaldi et al., 2000; Griffin and Mrak, 2002).

Recapitulating, we consider that neurodegenerative changes in AD are consequence of "mis-activated", dysfunctional microglia, proposing the "glia dysregulation hypothesis" (**Figure 6**; von Bernhardi, 2007). The innate immune response, normally protective, becomes abnormally activated, contributing to cytotoxicity (**Figure 5**; Nguyen et al., 2002; Wyss-Coray and Mucke, 2002; Saud et al., 2005; von Bernhardi, 2007). Normally activated microglia are important as the scavenger cells of the CNS. However, if they fail responding to their normal regulatory feedback and/or they show an impaired ability to clear Aβ (Paresce et al., 1997; von Bernhardi, 2007), glial cells could become predominantly cytotoxic. The distinction is relevant when developing therapeutic approaches. The aim of therapy should be oriented to potentiate a protective pattern of microglial cell function rather than functionally inhibiting microglia as it is most often proposed now.

Treatment Strategies for Neurodegenerative Diseases

Modulation of Microglial Cell Activation

Microglia are important actors for maintenance, repair and defense, although dysregulated microglia have deleterious effects. An effective microglia/neuroinflammation based therapy should target regulation of microglial cell response towards a beneficial pattern of activation, rather than their elimination. Because microglial function, as well as the deleterious effect of oxidative damage are associated with the activation of NADPH oxidase and the production of ROS that will act on both intracellular and extracellular targets (Block et al., 2006, 2007), this enzyme complex appears as a relevant therapeutic target. Originally linked only to respiratory burst in phagocytes, over the last decade it has been reported that NADPH oxidase homologues on diverse cells including neurons also play roles in normal function. Several peptides and small molecules, have been reported to inhibit NADPH oxidase, with potential neuroprotective effect over the last decade (Choi et al., 2005; Qin et al., 2005a). Because inhibition of NADPH oxidase activation targets the major generator of high amounts of ROS by microglia, its inhibition would reduce several inflammatory factors, including eicosanoids like PGE_2 (Wang et al., 2004). The challenge is to develop tools targeting NADPH oxidase isoforms responsible for overproduction of ROS by phagocytes like microglia. The efficacy as neuroprotector of the NADPH oxidase inhibitor diphenyleneiodonium has been reported in both LPS- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice (Wang et al., 2015). Diphenyleneiodonium attenuates progressive dopaminergic degeneration, with high efficacy in protecting the remaining neuronal population and restoring motor function even at late stages of disease progression in PD mouse models. Neuroprotection is associated with inhibition of microglial cell activation, decreased α-synuclein aggregation, and reduction of inflammatory mediators (Wang et al., 2015).

Also some inflammatory cytokines have been considered as possible therapeutic targets for AD (Greig et al., 2004; Heneka and O'banion, 2007; Lee et al., 2010). However, a side effect on therapies blocking inflammatory cytokines is the immune suppression caused by these drugs that leaves the patient prone to suffer grave infections. Systemic administration of the anti-inflammatory antibiotic minocycline, which inhibits microglia activation (Kohman et al., 2013a) affects strongly microglia, but also astrocytes, perivascular, meningeal, and infiltrating macrophages. It has been reported that minocycline restores LTP deficits, while normalizing the level of IL1β. These beneficial effects indicate that neuroinflammation could contribute to the deficits in synaptic plasticity, learning and memory observed during normal aging. However, minocycline use reveals the complexity of the effects of microglia function in neurodegenerative disease models. Minocycline show different effects on microglial cell activation and cognitive function along different phases of the life spans of animal models (Kohman et al., 2013a) suggesting that although inhibition of microglia can be beneficial at one stage of disease progression, it becomes detrimental at others.

Activation of Antioxidant Pathways

Reduction of ROS and oxidative stress could be also achieved through the activation of antioxidant pathways. In addition to the relatively weak antioxidant defenses of the brain, brain aging also determines loss of the endogenous mechanisms of free radical scavenging. Among cellular antioxidant defenses, heat shock proteins have been regarded as cytoprotector for oxidative damage-dependent mechanisms in neurodegenerative diseases. Among the stress proteins, the redox-regulated heme-oxygenase 1 (HO-1) gene, and its activation represents a protective system potentially active against brain oxidative injury. HO-1 polymorphisms have been associated with increased AD susceptibility, and dysregulation of the HO system has been associated with brain aging and the pathogenesis of AD (Markesbery, 1997; Pappolla et al., 1998). AD patients' brains present microglia recruitment by neurons with tau abnormalities. Those cell clusters correlate with increased levels of NRF2 and HO-1, suggesting an attempt of the diseased brain to limit microgliosis. Microglial cells HO-1 could be especially relevant for the regulation of neurotoxic mediators, being responsible of the antinflammatory effect of compounds such as schizandrin C (Park et al., 2013) and several other compounds (Foresti et al., 2013). Lastres-Becker et al. recently showed that fractalkine activated AKT in microglia, upregulating the transcription factor NRF2, and its target genes including HO-1. Fractalkine regulates microglial cell activation in neurodegenerative diseases. In a mouse model of tauopathy, they confirmed that NRF2- and fractalkine receptor-KO mice did not express HO-1 in microglia and showed they played a crucial role in the attenuation of neuroinflammation. Those observations suggest that NRF2-dependent induction of HO-1 could limit over-activation of microglia (Lastres-Becker et al., 2014). *In vitro* studies report a decreased HO-1 expression in HIV-infected macrophages. HO-1 deficiency correlates with increased glutamate and neurotoxicity, whereas HO-1 siRNA knockdown or its enzymatic inhibition in HIV-infected macrophages increased supernatant glutamate and neurotoxicity. In contrast, induction of HO-1 by dimethyl fumarate (DMF) decreased glutamate and neurotoxicity. Furthermore, increased IFNγ, as observed in CNS HIV infection, reduced HO-1 expression in cultured human astrocytes and macrophages (Gill et al., 2014). There are reports that activation of HO-1 is strongly protective against oxidative damage and cell death in neurons. Thus, modulation of HO-1 should represent a potential pharmaceutical strategy for the treatment of neurodegenerative disorders (Racchi et al., 2008; Schipper and Song, 2015).

Mitochondrial Antioxidants

Mitochondria have key roles in the production of ROS and in apoptosis signaling. Several compounds targeting mitochondria are currently being tested in clinical trials for treatment of neurodegenerative diseases. Mitochondrial antioxidants appear to be especially interesting at preclinical

level (Szeto et al., 2014). Coenzyme Q10 (CoQ10), a carrier of the electron transport chain of oxidative phosphorylation, has been shown to be neuroprotective by attenuating mitochondrial dysfunction and aging (Shetty et al., 2014). However, the fact that these oral antioxidants cross poorly the BBB, has slowed down their therapeutical use; directing new research towards more soluble, shorter chain CoQ10 derivatives, such as idebenone [6-(10-hydroxydecyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone], decylubiquinone (dUb), and MitoQ10. MitoQ10 has the advantage of being accumulated within mitochondria, where it is activated into ubiquinol, which can reduce mitochondrial oxidative damage (Lu et al., 2008). Other class of mitochondrial antioxidants are Szeto-Schiller (SS) peptides (Szeto, 2014), which localize in mitochondria at a broad condition of mitochondrial membrane potential. *In vivo* experiments revealed that SS peptides are protective, increasing survival and motor performance, and decreasing cell death (Moreira et al., 2010). In PD animal models, SS peptides also protect dopaminergic neurons against MPTP neurotoxicity (Moreira et al., 2010).

Therapeutic effects of the regulation of NADPH oxidase and antioxidant treatment will not be restricted exclusively to microglia. However, the development of drugs for specific isoforms and the fact that neuroinflammation is mostly driven by microglia and astrocytes, will have an enormous impact on the cytotoxic activation of glial cells, by reducing both ROS, inflammatory cytokines and endogenous inflammatory mediators.

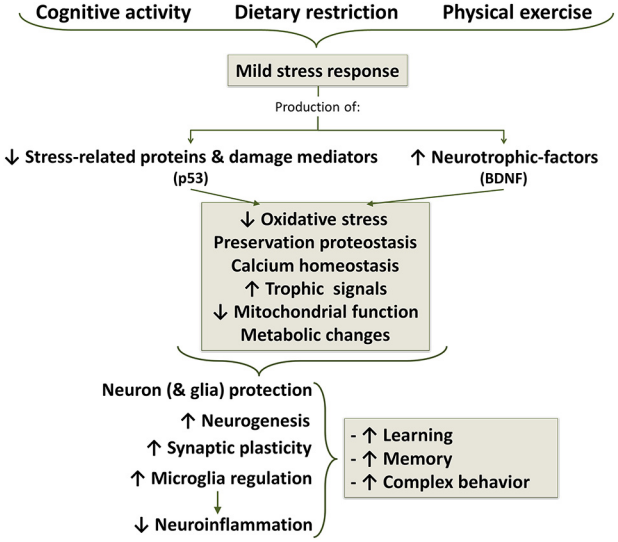

FIGURE 7 | Life style changes as a strategy for aging well. Cognitive activity, dietary caloric restriction and moderate physical exercise induce mild stress responses which results in a decreased production of stress proteins and reduction of oxidative stress. In additions, there is an increased production of neurotrophic factors, among which brain-derived neurotrophic factor (BDNF) appears to be one of the most important, but also participate growth hormone (GH) and insulin growth factor 1 (IGF1). Decreased stress signal and increased trophic signal acts on mitochondrial function, improving energetic metabolism and reducing oxidative stress to a protective level. Stress signals and ROS, below a certain threshold concentration, induce survival signals capable of restoring cellular homeostasis but, at higher or continued levels, can contribute to aging and degenerative changes.

Life-Style Changes Prevent Microglia dysrEgulation and Cytotoxic Activation

Accumulating evidence show that exercise, dietary restriction, cognitive intervention (enriched environment) as well as other mild stressors can play a role in reducing microglial activation and priming during aging (**Figure 7**). Moderate exercising is capable of even reducing the exaggerated neuroinflammation in response to infection-type of stimuli in aged animals, with its increased cytokine production and cognitive deficit (Barrientos et al., 2011), and age-related microglial sensitization (Barrientos et al., 2011; Kohman et al., 2013b), suggesting that exercise could be an effective intervention to prevent microglial cell aging. Furthermore, In adult APP/PS1 mice, exercise increase neurobehavioral performance, which is associated with increased numbers of certain populations of cholinergic and serotoninergic neurons, and reduced Aβ levels and microglia activation (Ke et al., 2011). Beneficial effects of exercise and cognitive intervention could, at least in part, result from its induction of brain-derived neurotrophic factor (BDFN; Barrientos et al., 2011; Polito et al., 2014). Although most of reports are related to the effect of BDNF on neuron function and survival, there are reports on its effect on inhibiting activation of microglia (Garofalo et al., 2015). Dietary restriction also appears to attenuate age-related activation of microglia, resulting in beneficial effects on neurodegeneration and cognitive decline (Morgan et al., 2007). It has anti-inflammatory and anti-apoptotic effects (Loncarevic-Vasiljkovic et al., 2012), and has been shown to elicit many health promoting benefits, delaying immunosenescence and attenuating neurodegeneration in animal models of AD and PD. However, the mechanisms involved in the effect of dietary restriction on microglial cell activation are poorly understood. Exposure to dietary restriction attenuates LPS-induced fever, and LPS-induced microglial activation in some specific brain regions, including the arcuate and ventromedial nuclei of the hypothalamus and the subfornical organ. Activation of microglia in the hypothalamic nuclei was positively correlated with body temperature (Radler et al., 2014). Dietary restriction suppresses LPS-induced secretion of inflammatory cytokines, and shifts hypothalamic signaling pathways to an anti-inflammatory bias (Radler et al., 2015).

Interestingly, both exercise and dietary restriction have been recently shown to promote mitochondrial biogenesis and expression of mitochondrial transcription factor A (TFAM) in the rat brain (Picca et al., 2012; Zhang et al., 2012). Collectively, exercise, cognitive activity, and dietary restriction could be effective ways to slowdown brain aging by preventing microglia aging through secretion of growth factor and regulatory cytokines. Although those effects are not restricted to microglia, the fact that microglia are the major drivers of neuroinflammation, determines that interventions affecting them can have an enormous impact on the brain homeostatic response.

Concluding Remarks

Aging is a major risk factor for the great majority of neurodegenerative diseases. Age dependent changes, including increased glial cell activation, neuroinflammation, oxidative stress, impaired mitochondrial function, and impaired protein processing, could lead to the dysregulation of microglial cell functions resulting, among several alterations in cytotoxicity and accumulation of Aβ, generating the hallmark histopathology of AD. Whereas each of these age-dependent changes are discreet in the normal aging process, their combined effect, together with the genetic background and environmental conditions could initiate the vicious circle of cytotoxic activation (von Bernhardi, 2007). Participation of oxidative stress could be both a trigger and a consequence of Aβ accumulation, mitochondrial impairment, cytotoxic activation of microglia, proteasome dysfunction and protein misfolding, contributing to the potentiation of the other disease mechanisms. Additionally, oxidative stress, cytotoxicity and Aβ aggregation further decrease proteasome activity, creating a vicious circle leading to more Aβ and tau aggregation.

Microglia, in a close crosstalk with astrocytes, neurons and other brain cells, serve crucial functions as the scavenger system of the CNS, providing beneficial functions as tissue repair in the CNS. However, chronic, dysregulated activation of microglia appears to lead to deleterious effects inducing malfunction and damage of brain cells. What drives this dysregulation is not fully understood, but age-related impairment of regulatory mechanisms, as observed for TGBβ transduction signaling (Tichauer et al., 2014) are a promising hypothesis for understanding cytotoxic activation in aged individuals (von Bernhardi et al., 2011). Nonetheless, despite the undeniable potential of activated microglia to become deleterious, microglia have a profound immune-modulatory and reparative potential in the CNS. Thus, instead of abolishing microglia activation as it is most often proposed, strategies to potentiate those beneficial functions while inhibiting cytotoxic activation should be developed. Such strategy may well constitute the way to treat neurodegenerative disorders, but demands a better understanding of the protective and modulatory pathways of immune activation. Additional research is needed for the identification of new pathways that may decrease the impact of microglial cell dysfunction, in order of breaking the vicious circle leading to neurotoxicity.

Further research is necessary to develop effective pharmacological interventions against brain aging. Most of the proposed targets, antioxidants, anti-inflammatory drugs affecting cytokines, and microglia inhibitors, deeply affect physiological cell signaling and functions, including pro-survival signaling pathways, resulting in unacceptable side effects. In that perspective, multi-target pharmacological approaches aimed to reestablish normal regulation of microglia in the aged brain may be future research avenue for slowing senescence-related impairment. Furthermore, non-pharmacological strategies, like exercise, life style changes and dietary restriction, could promote a healthy aging through their effects on promoting microglial physiological functions, while reducing inflammation and ROS production.

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Oxidized cholesterol as the driving force behind the development of Alzheimer's disease

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Alzheimer's disease (AD), the most common neurodegenerative disorder associated with dementia, is typified by the pathological accumulation of amyloid Aβ peptides and neurofibrillary tangles (NFT) within the brain. Considerable evidence indicates that many events contribute to AD progression, including oxidative stress, inflammation, and altered cholesterol metabolism. The brain's high lipid content makes it particularly vulnerable to oxidative species, with the consequent enhancement of lipid peroxidation and cholesterol oxidation, and the subsequent formation of end products, mainly 4-hydroxynonenal and oxysterols, respectively from the two processes. The chronic inflammatory events observed in the AD brain include activation of microglia and astrocytes, together with enhancement of inflammatory molecule and free radical release. Along with glial cells, neurons themselves have been found to contribute to neuroinflammation in the AD brain, by serving as sources of inflammatory mediators. Oxidative stress is intimately associated with neuroinflammation, and a vicious circle has been found to connect oxidative stress and inflammation in AD. Alongside oxidative stress and inflammation, altered cholesterol metabolism and hypercholesterolemia also significantly contribute to neuronal damage and to progression of AD. Increasing evidence is now consolidating the hypothesis that oxidized cholesterol is the driving force behind the development of AD, and that oxysterols are the link connecting the disease to altered cholesterol metabolism in the brain and hypercholesterolemia; this is because of the ability of oxysterols, unlike cholesterol, to cross the blood brain barrier (BBB). The key role of oxysterols in AD pathogenesis has been strongly supported by research pointing to their involvement in modulating neuroinflammation, Aβ accumulation, and cell death. This review highlights the key role played by cholesterol and oxysterols in the brain in AD pathogenesis.

Keywords: Alzheimer's disease, oxidative stress, inflammation, oxidized cholesterol, oxysterols

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Abbreviations: α-EPOX, 5α, 6α-epoxicholesterol; β-EPOX, 5β, 6β-epoxicholesterol; 24-OH, 24-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 4β-OH, 4β-hydroxycholestrerol; 7β-OH, 7β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 7-OH-4-C, 7α-hydroxy-3-oxo-4-cholestenoic acid; 7-K, 7-ketocholesterol; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; AD, Alzheimer's disease; ApoE, apolipoprotein E; APP, amyloid precursor protein; Aβ, amyloid β; BACE1, beta-site amyloid precursor protein cleaving enzyme 1; BBB, blood-brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; CYP27A1, 27-hydroxylase; CYP46A1, 24-hydroxylase; LPS, lipopolisaccaride; LXR, liver X receptor; NF-κB, nuclear factor-κB; NFT, neurofibrillary tangles; NO, nitric oxide; PSEN1, presenilin 1; PSEN2, presenilin 2; RNS, reactive nitrose species; ROS, reactive oxygen species; TLR, Toll-like receptor.

Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder associated with dementia, and the progressive deterioration of mental capacities. It is a complex disease characterized by progressive memory impairment, cognitive deficit, and personality changes; these symptoms are due to substantial synaptic and neuronal loss occuring in specific brain areas, especially in the neocortex, hippocampus, and other subcortical regions. Brains from AD patients show distinct neuropathological features, which we now know are the hallmarks of the disease: extracellular deposits of amyloid β (Aβ) peptides in the form of senile plaques, Aβ deposits in the cerebral blood vessels, and intracellular inclusion of neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein (Querfurth and LaFerla, 2010; Chopra et al., 2011).

One of the events that promotes AD pathogenesis is the abnormal processing of amyloid precursor protein (APP), which leads to excess production of Aβ peptides through the sequential enzymatic actions of beta-site APP cleaving enzyme 1 (BACE1), a β-secretase, and γ-secretase, both enzymes of the amiloidogenic pathway. An imbalance between the production and clearance of Aβ peptides in the brain, and their aggregation, cause Aβ to accumulate, with subsequent formation of senile plaques. Depending on the site of γ-secretase cleavage, two major forms of Aβ are generated: a peptide of 40 amino acids ($A\beta_{1-40}$), and one of 42 amino acids ($A\beta_{1-42}$). $A\beta_{1-42}$ is the predominant species of Aβ in senile plaques, and the insoluble oligomers and intermediate amyloids are its most neurotoxic forms (Walsh and Selkoe, 2007). Aβ oligomers are hypothesized to cause neuronal damage and cognitive failure by generating free radicals, as well as mitochondrial oxidative damage, synaptic failure, and inflammatory changes in AD brains (Oddo et al., 2003; Mattson, 2004; Reddy and Beal, 2005; Castellani et al., 2010). Different assembly states of Aβ, and its accumulation in different cellular compartments, can then affect critical pathways, thereby facilitating the development of tau pathology. Experimental evidence confirms that Aβ accumulation precedes and drives NFT formation (Götz et al., 2001; Lewis et al., 2001).

Besides aging, which is the most obvious risk factor for the disease, a number of theories point to other risk factors, such as culture, lifestyle, head injury, and genetics. Other risk factors are associated with vascular disease, including hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes (Mayeux, 2003). Some evidence suggests that the dietary intake of homocysteine-related vitamins (vitamin B12 and folate), antioxidants (vitamins C and E), unsaturated fatty acids, and also a moderate alcohol intake, especially in the form of wine, may reduce the risk of AD (Luchsinger and Mayeux, 2004).

Although environmental factors increase the risk of AD, this disease can also be caused by various gene mutations (Gatz et al., 2006). In this contest, mutation in the genes *APP*, presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) accounts for most cases of the familial (or early-onset) form of AD, by increasing the production and aggregation of Aβ and amyloid plaque formation (Tanzi and Bertram, 2005). Conversely, the apolipoprotein E (*ApoE*) gene is an important genetic risk factor for the sporadic (or late-onset) form of AD (Raber et al., 2004). The contribution of other candidate genes is probably less important, and none has been verified. A possible explanation for this difficulty in clarifying the genetic background might be that the sporadic form of AD is not a uniform disease entity, and that several susceptibility-enhancing genes may act in concert, each conferring only a small increase in risk, in a complex interaction with environmental factors.

However, although the pathophysiology of AD is still not clearly understood, considerable evidence indicates that many events participate in the development and progression of the disease, including oxidative stress, inflammation, glial cell activation, dysregulation of metal ions and calcium, presence of ApoEε4, altered cholesterol metabolism, and dysregulation of intercellular communication among brain cells (Quintanilla et al., 2012).

Oxidative Stress in AD Pathogenesis

It has been extensively reported that free radicals are pathologically important in neurodegenerative diseases, and that the brain tissue is exposed to oxidative damage during the development of AD, already from its early onset (Smith et al., 2000, 2010; Mariani et al., 2005; Zhu et al., 2005; Reynolds et al., 2007). Because age is a significant risk factor for AD, it is also widely accepted that oxidative stress increases with age leading to the accumulation of oxidative damage in biomolecules (Butterfield and Kanski, 2001; Martin and Grotewiel, 2006; Jacob et al., 2013).

The brain is particularly vulnerable to oxidative damage for numerous reasons, but chiefly because it utilizes about 25% of the respired oxygen, with a consequent increase of free radicals; it also contains high concentrations of catalytic iron and lipids, which are easily oxidized by free radicals. Further, the brain contains relatively low levels of antioxidants and antioxidant defense enzymes, and is thus not very efficient at removing free radicals (Ansari and Scheff, 2010; Mazzetti et al., 2015). Because the brain has a high lipid content, it is extremely vulnerable to oxidative species, with the consequent enhancement of lipid peroxidation and cholesterol oxidation, and the subsequent formation of end products, mainly 4-hydroxynonenal and oxysterols, respectively from the two processes (Sottero et al., 2009; Reed, 2011).

Within the brain, neurons are the cells most vulnerable to excess reactive oxygen species (ROS) and reactive nitrose species (RNS), and their survival depends on the antioxidant action of astrocytes. Astrocytes are very important for normal brain function, because of their ability to actively promote neuroprotection, in particular by releasing glutathione, which protects neurons from oxidative stress (Shih et al., 2003). However, neurons can also defend themselves through an intrinsic mechanism of antioxidant defense involving the glucose metabolism (Fernandez-Fernandez et al., 2012).

AD brains display high levels of oxidative stress, and a direct association between free radical generation and the presence of

Aβ plaques has been shown both in living AD mouse models and in human AD tissue (McLellan et al., 2003). However, it is still not clear whether oxidative stress is a cause or a consequence of the neuropathology associated with AD (Zhu et al., 2007; Bonda et al., 2010; Smith et al., 2010; Luque-Contreras et al., 2014). In support of its being a cause, it has been proposed that oxidative stress precedes the onset of clinical and pathological AD symptoms, including Aβ deposition, NFT formation, vascular malfunction, and cognitive decline (Nunomura et al., 2001; Praticò et al., 2001). In this connection, in a triple-transgenic mouse model of AD, mimicking AD progression in humans, the levels of antioxidants (glutathione and vitamin E) were found to be decreased, and the extent of lipid peroxidation increased, before the appearance of senile plaques and NFT (Resende et al., 2008). Moreover, as a prominent early event, oxidative stress is believed to contribute to tau hyperphosphorylation in neurons (Su et al., 2010) and, of note, it has been observed that neuritic and cored amyloid plaques show evidence of oxidatively modified Aβ (Head et al., 2002). Conversely, Aβ is a potent promoter of oxidative stress, since it is a potent generator of both ROS (Ding et al., 2007) and RNS (Combs et al., 2001). Within the Aβ sequence, it has been suggested that methionine 35 plays an important role in promoting oxidative activity. When this amino acid is replaced by cysteine, the oxidative stress induced by Aβ is greatly attenuated (Butterfield and Boyd-Kimball, 2005; Butterfield et al., 2013). In this connection, it has also been proposed that the amyloid oligomers may insert themselves into the lipid bilayer, causing lipid peroxidation and, consequently, oxidative damage to proteins and other biomolecules (Butterfield et al., 2001). Aβ oligomers are, indeed, the strongest inducers of oxidative stress among all Aβ species (Tamagno et al., 2006; Naylor et al., 2008). Generation of free radicals, altered membrane properties, as well as disturbed calcium homeostasis, may also underlie the apoptotic effect of Aβ oligomers (Malaplate-Armand et al., 2006). It has also been observed, not only in cultured neurons but also *in vivo* using the double transgenic model of AD (APP/PS1), that Aβ causes an increase in oxidative stress that leads to phosphorylation of p38, which in turn phosphorylates tau at its T231 residue (Giraldo et al., 2014).

Mitochondrial dysfunction is another feature of AD pathogenesis (Castellani et al., 2002). Defects in the mitochondria are typically defects of the electron transport chain; these contribute both to the hyperproduction of a variety of ROS, and to the deficiency of several key enzymes responsible for oxidative metabolism that, in turn, cause cell damage and eventual death (Cottrell et al., 2001). Moreover, it has been shown that oxidative species, through mitochondrial impairment, cause tau hyperphosphorylation leading to neuron and synapse loss (Melov et al., 2007). Mitochondrial dysfunction and oxidative damage have been investigated in triple-transgenic mice that develop both Aβ and tau disorders. These mice exhibited increased oxidative stress, manifested by increased hydrogen peroxide production and lipid peroxidation (Yao et al., 2009; Reddy, 2011).

APP and Aβ have also been associated with dysfunctional consequences for mitochondrial homeostasis and cell death (Manczak et al., 2006). APP impairs mitochondrial energy metabolism, thus causing mitochondrial abnormalities leading to ROS production (Anandatheerthavarada et al., 2003); accumulation of APP in the mitochondrial import channel then potentially inhibits mitochondrial import (Reddy et al., 2004). Aβ is also considered a potent mitochondrial poison, especially affecting the synaptic pool. It appears that Aβ enters the mitochondria, induces free radical generation, disrupts the electron transport chain, and ultimately causes mitochondrial dysfunction (Mungarro-Menchaca et al., 2002). Aβ, then, inhibits key mitochondrial enzymes in the brain and in isolated mitochondria (Caspersen et al., 2005; Reddy and Beal, 2008), and cytochrome c oxidase is also specifically attacked (Crouch et al., 2005). A recent study on the transgenic mouse brain confirmed that Aβ accumulates in neuronal mitochondria, thus affecting mitochondrial function, as shown by increased mitochondrial permeability, the decline of both respiratory function and cytochrome c oxidase activity, and increased mitochondrial oxidative stress (Du et al., 2010).

An association between ApoEε4 and oxidative stress-mediated damage in AD has been also suggested. Despite playing a beneficial role, by maintaining lipid homeostasis and redox balance, ApoE can also contribute to oxidative damage in an isoform-dependent manner, the ApoEε4 isoform being the most harmful in AD (Luque-Contreras et al., 2014). The *ApoEε4* genotype is also involved in mitochondrial dysfunction (Chang et al., 2005), and might be a risk for potential antioxidant system loss in AD (Shea et al., 2002).

Alterations in cerebrovascular regulation have recently been ascribed to the early stages of AD, and the vascular endothelium is also a target for oxidative stress leading to endothelium dysfunction (Iadecola, 2004; Park et al., 2008). Chronic hypoperfusion may thus play an important role in the pathophysiology of AD, because it induces oxidative stress, and over time this damage could initiate mitochondrial failure (Sochocka et al., 2013). A recent study has shown that inhibition of NADPH oxidase activity can mitigate cognitive impairment in rodent models of hypoperfusion (Kim et al., 2012). Oxidative stress is intimately associated with neuroinflammation, and there has been found to be a vicious circle connecting oxidative stress and inflammation in AD (Rosales-Corral et al., 2010; Quintanilla et al., 2012; Joshi and Praticò, 2015). It has been observed that the redox status modulates inflammatory factors involvement in signaling processes, which are critical mediators of oxidative stress and inflammation, causing neurodegeneration (Mrak and Griffin, 2005; Kierdorf et al., 2010). Activation of glial cells and increased cytokine production is also induced by oxidative stress and, in turn, glial activation leads to the release of other neurotoxic factors such as ROS and nitric oxide (NO), which further exacerbate neuronal damage (Town et al., 2005; Block et al., 2007; Michelucci et al., 2009; von Bernhardi et al., 2010). Consequently, the resultant cellular damage amplifies the inflammatory response, with glial activation and leukocyte recruitment, leading to further inflammation in the AD brain (**Figure 1**).

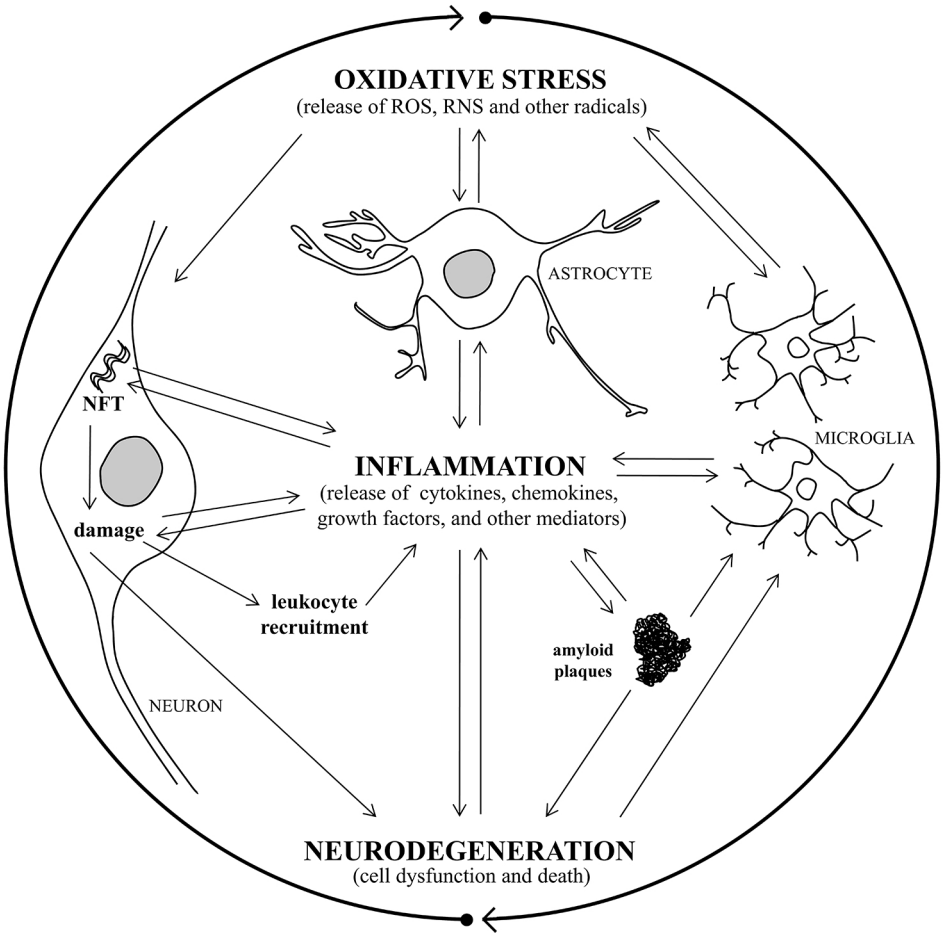

FIGURE 1 | A vicious circle connects oxidative stress, inflammation, and neurodegeneration in Alzheimer's disease (AD). Oxidative stress damage and inflammatory response are closely associated with AD, causing neurodegeneration. Oxidative stress induces activation of microglia and astrocytes with a consequent increase of pro-inflammatory mediator production and, in turn, glial activation leads to toxic radical release, exacerbating neuronal damage. Consequently, the resultant cellular damage amplifies the inflammatory response, with glial activation and leukocyte recruitment, leading to further inflammation in the AD brain. The release of inflammatory cytokines leads to amyloid plaque and neurofibrillary tangle (NFT) formation, that triggers inflammatory molecule release and causes neuronal damage, with consequent neurodegeneration.

Inflammation in AD Pathogenesis

Besides oxidative stress damage, inflammatory responses are also closely associated with AD pathology. Inflammation occurs in pathologically vulnerable regions of the AD brain, with increased expression of acute-phase proteins and pro-inflammatory molecules, which create a chronic and self-sustaining inflammatory process, involving activated glia cells, and stressed neurons (Mrak and Griffin, 2005; Perry et al., 2010; Morales et al., 2014). Thus chronic inflammation plays a basic role in the progression of neuropathological changes in AD, resulting in neuronal dysfunction and death. The importance of neuroinflammation has emerged from several studies of AD brains, which have evidenced the activation and proliferation of glial cells, together with enhanced release of inflammatory mediators (cytokines, chemokines, growth factors, and other mediators) and free-radical-mediated oxidative stress (ROS, NO, and other radicals) (Glass et al., 2010; Heneka et al., 2010; McGeer and McGeer, 2010; Holmes and Butchart, 2011; Azizi and Mirshafiey, 2012; Rubio-Perez and Morillas-Ruiz, 2012; Lyman et al., 2014; Figueiredo-Pereira et al., 2015). Microglia are key players in the disease process, and once activated they present cell-surface antigens commonly present on monocytes and macrophages (Latta et al., 2014). Microglial activation thus leads to the initiation of an innate immune response, dominated by the release of pro-inflammatory cytokines (Town et al., 2005; Mrak, 2012; Weitz and Town, 2012). Incidental to this is also the phagocytosis of fibrils and large aggregates of Aβ, suggesting an initial neuroprotective defense mechanism (D'Andrea et al., 2004; Colton and Wilcock, 2010). Further, microglia can also secrete a number of soluble factors, such as glia-derived neurotrophic factor, which are potentially beneficial to the survival of neurons (Liu and Hong, 2003). Although the

initial purpose of microglial activation is to counteract the detrimental effects induced by the pathological features, it subsequently leads to the release of high concentrations of neurotoxic factors, such as inflammatory molecules, NO, ROS, proteolitic enzymes, complementary factors, and excitatory amino acids, which further exacerbate cell damage (Michelucci et al., 2009). Moreover, in later stages of the disease, the overproduction of inflammatory cytokines makes the microglia phagocytically inactive, especially against insoluble oligomers and high concentrations of Aβ (Hickman et al., 2008; Krabbe et al., 2013).

A growing body of evidence also suggests that the central nervous system (CNS) and systemic inflammation cannot be viewed in isolation. Systemic inflammation might exarcerbate behavioral symptoms and accelerate AD progression by increasing the production of local pro-inflammatory cytokines and chemokines, as well as of ROS and NO (Holmes, 2013). The detrimental effects of peripheral inflammatory molecules in the brain of AD patients chiefly occur because these mediators can easily enter the brain, together with infiltrating leukocytes, thanks to increased permeability of the blood-brain barrier (BBB) as the disease progresses (Leoni et al., 2003; Popescu et al., 2009; Takeda et al., 2014). Attention is also now being paid to the participation of Toll-like receptors (TLRs) in inflammation and neurodegeneration. The recruitment of TLRs contributes to inflammation by amplifying the release of inflammatory molecules, thus playing an important role in the impact of inflammation on neuronal function and death (Drouin-Ouellet and Cicchetti, 2012).

However, it also remains unclear in the case of inflammation whether this process is a cause or a consequence of AD. Clinical and experimental studies support the appearance of neuroinflammatory changes already at the early stages of AD, even before the formation of extracellular Aβ deposits and intracellular NFT accumulation (Sheng et al., 2000). The release of pro-inflammatory cytokines and enzymes could affect the normal behavior of neuronal cells, leading to cell dysfunction and abnormalities such as Aβ peptides and NFT accumulation, events in the pathway leading to neuronal degeneration. Inflammatory molecules, and a number of stress conditions, enhance APP levels and the amyloidogenic processing of APP to induce $A\beta_{1-42}$ peptide production. This, in turn, inhibits the formation of the soluble APP fraction that seems to have a neuronal protective effect (Fassbender et al., 2000; Misonou et al., 2000). Conversely, it has been demonstrated that intraneuronal Aβ and soluble Aβ oligomers activate microglia in the earliest stages of AD, even before senile plaque and NFT formation, in particular when cells are stressed (Ferretti and Cuello, 2011; Khandelwal et al., 2011). Moreover, Aβ and NFT have also been shown to trigger the release by activated glia cells of pro-inflammatory mediators, free radicals, and other neurotoxic substances, implying that the development of AD leads to the initiation of several self-propagating cycles (Vukic et al., 2009; Morales et al., 2013). Fibrillar Aβ can also activate microglia by binding to cell membranes via specific receptors, including a multi-receptor complex involving CD36, $\alpha_6\beta_1$-integrin, and CD47 (Verdier et al., 2004; Yu and Ye, 2015), while the disruption of the *APP* gene and of its proteolytic products delays and decreases microglial activation (DeGiorgio et al., 2002). Once activated, microglia may also recruit and activate astrocytes, which actively enhance the inflammatory response to extracellular Aβ deposits (Jo et al., 2014). Aggregated amyloid fibrils and neurotoxic inflammatory molecules, secreted by glial cells, intensify neuronal dysfunction and death, either alone or in concert (Brown and Bal-Price, 2003; Findeis, 2007). In this context, activated glia cells and the released inflammatory molecules, together with other components of the immune response, are often present in proximity to neurons and areas of amyloid plaque (Abbas et al., 2002; Serrano-Pozo et al., 2013). Of note, microglia have also been suggested to be preferentially associated with certain types of amyloid deposits (D'Andrea et al., 2004). Furthermore, astrocytes are known to play a critical role in Aβ clearance, in providing trophic support to neurons, and in forming a protective barrier between Aβ deposits and neurons. The presence of large numbers of astrocytes associated with Aβ plaques suggests that these lesions induce the release of chemotactic molecules that mediate astrocyte recruitment. However, it has been suggested that astrocytes could also be a source for Aβ peptides, because they overexpress BACE1 in response to chronic stress (Roßner et al., 2005; Wang et al., 2011). While Aβ has been widely demonstrated to be pro-inflammatory, the association between microglial activation and tau pathology development is still not supported by direct evidence, since neurofibrillary disorder occurs both in the presence and absence of neuroinflammation (Streit et al., 2014), and intraneuronal NFT lesions usually precede the formation of Aβ aggregates (Braak and Del Tredici, 2004).

Although it has been reported that microglia and astrocytes actively promote disease development, and play pivotal roles in amyloid deposition (Wegiel et al., 2004), conversely it has also been reported that microglia are protective and may remove amyloid deposits, thus having no effect on AD development (Simard et al., 2006; Grathwohl et al., 2009). Moreover, and interestingly, it has been suggested that infiltrating macrophages from the circulation, rather than microglia, play a central role in clearing Aβ deposits in cerebral amyloid angiopathy (Hawkes and McLaurin, 2009), but that these cells can also play a determinant role in AD development (Gate et al., 2010; Rezai-Zadeh et al., 2011).

Of note, while neurons were traditionally believed to be passive bystanders in neuroinflammation, some recent evidence suggests that not only astrocytes and microglia, but also neurons themselves, contribute to the chronic inflammation in AD, by serving as a source of inflammatory molecules (Tchelingerian et al., 1994; Yan et al., 1995; Murphy et al., 1999; Suzuki et al., 1999; Acarin et al., 2000; Heneka and O'Banion, 2007; Rubio-Perez and Morillas-Ruiz, 2012; Ramesh et al., 2013). An increase in pro-inflammatory molecule expression has also been observed in human neuroblastoma SH-SY5Y cells after incubation with some cholesterol oxidation products (known as oxysterols) potentially implicated in AD pathogenesis (Testa et al., 2014).

Brain Cholesterol Metabolism and AD

The human brain contains approximately 25% of the body's cholesterol; this is essential for its normal functioning, being a major component of neuronal cell membranes and an essential factor in membrane fluidity. In the adult brain, cholesterol is mostly present in its non-esterified form; however, small amounts of desmosterol and cholesteryl ester are also present. The brain is separated from the peripheral circulation by the BBB, which prevents the dietary intake of cholesterol being transported from the circulation to the brain, since lipoproteins do not cross the BBB. This means that nearly all the brain cholesterol is synthesized *de novo* within the CNS, from 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, through a complex series of reactions involving more than 20 enzymes; cholesterol metabolism is, thus, regulated independently of that in the peripheral tissues (Vance et al., 2005; Dietschy, 2009). Because neurons do not efficiently synthesize cholesterol, they rely on astrocytes as an external source. Astrocytes meet neuronal cholesterol demands by secreting ApoE-cholesterol complexes, which are transported to the neurons for their development and function. On note, ApoE transcription is regulated by 24-hydroxycholesterol (24-OH) released by the neurons via the liver X receptor (LXR), this cholesterol oxidation product being one of its natural ligands (Pfrieger, 2003; Nieweg et al., 2009). LXR is a nuclear receptor that regulates the expression of specific genes involved in cholesterol efflux and metabolism, such as ATP-binding cassette transporters A1 and G1 (*ABCA1* and *ABCG1*), and *ApoE* (Sodhi and Singh, 2013).

ApoE is the brain's principal cholesterol-carrier protein; through its receptors it regulates the redistribution and homeostasis of cholesterol within the brain. Humans express three naturally-occurring alleles of the *ApoE* gene: ε2, ε3, and ε4. Murine studies have shown that astrocytes and microglia are the primary ApoE secreting cells in the brain, but neurons can express ApoE under conditions of excitotoxic injury (Xu et al., 2006).

The association between ApoE polymorphism and AD is presumably related to disorders in cholesterol transport. In this connection, it has been found that receptors recognizing ApoE are widely expressed in the brain of AD patients. Of note, AD is accelerated in ApoEε4 individuals: those who are homozygous for the ε4 allelic variant of ApoE have a 50–90% higher chance of developing AD by age 85 years than those carrying ε2 and ε3, showing that this ApoE isoform is one of the major risk factors for AD (Puglielli et al., 2003; Evans et al., 2004; Bu, 2009; Kim et al., 2009a; Martins et al., 2009; Schipper, 2011). However, the mechanisms whereby ApoE isoforms affect the risk of AD are still obscure. One hypothesis is that ApoEε4 protein accelerates the uptake of cholesterol-rich particles by the vasculature, leading to more rapid disease progression: compared to ApoEε4, ApoEε2 and ApoEε3 proteins show reduced receptor binding.

Although ApoE facilitates intracellular Aβ degradation by microglia, reducing intracellular cholesterol levels (Lee et al., 2011) by accelerating its clearance by binding Aβ and forming a stable complex (Sagare et al., 2007; Jiang et al., 2008), ApoE is, conversely, essential for Aβ aggregation and deposition, promoting Aβ fibrillization and plaque formation, as well as tau hyperphosphorylation and NFT formation (Cam and Bu, 2006; Bu, 2009; Marzolo and Bu, 2009). Of note, in the AD-affected brain, ApoE colocalizes with cholesterol and fibrillar Aβ in senile plaques (Burns et al., 2003b). It has also been shown that the *ApoEε4* genotype enhances Aβ production and Aβ fibril formation *in vitro*, as well as in transgenic mice with mutated APP, more than in mice expressing ApoEε3 (Holtzman et al., 2000; Carter et al., 2001; Ye et al., 2005). In addition, ApoEε4 synergizes with Aβ toxicity (Ji et al., 2002). Conversely, a striking reduction in amyloid deposits has been observed in all brain regions of ApoE-null mice (Bales et al., 1999). It has also been suggested that, in AD patients with ApoEε4, a decreased ability to clear Aβ contributes to increasing Aβ accumulation and amyloid plaque formation (Deane et al., 2008). In addition, ApoE may mediate Aβ cell internalization, by binding to the low density lipoprotein receptor-related protein (LRP; Herz and Beffert, 2000). Further, like APP, ApoE also undergoes cleavage, and ApoEε4 is more susceptible to cleavage than is ApoEε3. Fragments of ApoE, such as Aβ, can be toxic, causing AD-like neurotoxicity in mouse models (Harris et al., 2003; Brecht et al., 2004); the lipid-binding region of ApoE is required for this toxicity (Chang et al., 2005).

Accumulating evidence also supports a key role for human ApoE in modulating neuroinflammation (Maezawa et al., 2006c; Keene et al., 2011; Tai et al., 2015). Many studies have reported that ApoEε4 induces a detrimental neuroinflammatory phenotype, both in peripheral and CNS inflammation. *In vitro* data, using microglia isolated from ApoE transgenic mice, has demonstrated *ApoE* genotype-specific modulation of TLR4/lipopolisaccaride (LPS) induced inflammation. In particular, LPS-induced pro-inflammatory cytokines are more strongly expressed in ApoEε4 and ApoEε3 (Maezawa et al., 2006b). Moreover, in microglia treated with LPS/interferon γ, pro-inflammatory cytokine levels were higher, and anti-inflammatory cytokine levels were lower, in ApoEε4 compared to ApoEε3. Unlike what occurs in microglia, in astrocytes TLR4/LPS-induced secretion of pro-inflammatory cytokines follows the pattern: ApoEε2 > ApoEε3 > ApoEε4 via differential nuclear factor-κB (NF-κB) activation (Maezawa et al., 2006a). With regard to ApoE modulation of Aβ-induced neuroinflammation, the available results are scarse. It has been demonstrated that impaired ApoEε4 function modulates the effects induced by Aβ on inflammatory receptor signaling, by amplifying the detrimental pathway TLR4-p38α and suppressing the beneficial pathway interleukin-4R-nuclear receptor (Tai et al., 2015). Importantly, ApoE also modulates BBB function, by a process that may be considered neuroinflammatory. It has been observed that a lack of murine ApoE combined with the expression of ApoEε4, but not of ApoEε2 nor ApoEε3, leads to BBB breakdown by activating the pro-inflammatory pathway cyclophilin A-NF-κB-matrix metalloproteinase 9 in pericytes. Consequently, blood-derived neurotoxic proteins are taken up by neurons,

and microvascular and cerebral blood flows are reduced. It has been shown that the vascular defects in ApoE-deficient and ApoEε4-expressing mice precede neuronal dysfunction and can initiate neurodegenerative changes (Bell et al., 2012).

In addition to ApoE, other LXR-responsive genes involved in cholesterol efflux are the *ABCA1* and *ABCG1* (Voloshyna and Reiss, 2011). ABCA1 is reported to be involved in ApoE metabolism and Aβ production, as well as in the modulation of amyloid plaque formation in the CNS (Koldamova et al., 2010; Kim et al., 2011). A close correlation between Aβ and ABCA1 levels has been demonstrated, since, in cultured astrocytes, Aβ inhibits ABCA1 expression (Canepa et al., 2011). In addition, it has also been shown that AD transgenic mice lacking ABCA1 develop increased Aβ levels and senile plaques, in the absence of changes in APP processing (Wahrle et al., 2004). By contrast, in transgenic mice overexpressing ABCA1, the increased ABCA1 function significantly decreases Aβ deposition (Wahrle et al., 2008). Another ABC transporter, ABCA7, has been found to stimulate cellular cholesterol efflux to ApoE-containing particles in the same way as ABCA1 (Chan et al., 2008).

Cholesterol transport and homeostasis are thus closely linked to multiple aspects of Aβ biology, since cholesterol levels influence the production and deposition of the pathogenic Aβ peptides (Burns et al., 2003a; Hughes et al., 2014; Lane-Donovan et al., 2014). Cholesterol has indeed been shown to directly modulate the processing of APP in neuronal cell cultures (Ehehalt et al., 2003), probably by promoting β-secretase activity (Xiong et al., 2008). However, the mechanisms whereby cholesterol affects Aβ production and deposition are still not fully understood. A change in membrane properties and distribution of cholesterol has been suggested as a possible mechanism (Shobab et al., 2005). Cholesterol is mainly concentrated in membrane microdomains termed lipid rafts, which are considered to be the site of the amyloidogenic pathway (Cordy et al., 2006; Vetrivel and Thinakaran, 2010). Further, it has been shown that cellular cholesterol, especially when levels in the membrane are elevated, binds directly to APP at its C terminal transmembrane domain (Harris, 2008; Beel et al., 2010); as a consequence of the binding APP is inserted into the phospholipid monolayers of the lipid rafts and other organelles, where β- and γ-secretases reside (Wahrle et al., 2002; Beel et al., 2010). The amyloidogenic pathway of APP processing is thus linked to cholesterol levels in these microdomains: β- and γ-secretase activities are stimulated by high, and inhibited by low levels of cholesterol (Grimm et al., 2008; Xiong et al., 2008). Conversely, in the non-amyloidogenic pathway, APP is processed by α-secretase in non-raft domains, and this event is promoted by a decreased cellular cholesterol level (Reid et al., 2007). Moreover, α-secretase can be forced to associate with lipid rafts thus inactivating the amyloidogenic pathway (Harris et al., 2009). Of note, it has also been suggested that high levels of non-esterified cholesterol alone might not affect APP processing, while the conversion of free cholesterol to esterified cholesterol might up-regulate APP processing and Aβ generation (Puglielli et al., 2001). Conversely, inhibition of the enzyme acyl-coenzyme A:cholesterol acyl-transferase 1, which esterifies cholesterol, leads to the formation of smaller amounts of cholesteryl esters and Aβ (Bhattacharyya and Kovacs, 2010). These observations suggest that the balance between non-esterified and esterified cholesterol is a fundamental point controlling the amyloidogenic pathway.

It has also been shown that increased cholesterol levels in the lipid bilayers favor Aβ binding to cell membranes (Kakio et al., 2001). Additionally, cholesterol interacts with the peptide as soon as it inserts into the lipid bilayer, and accelerates its recruitment and oligomerization (Fantini and Yahi, 2010). Indeed, cholesterol enhances Aβ to form neurotoxic aggregates (Yanagisawa, 2005), and binds avidly to Aβ protofibrils at level of lipid rafts where fibrillogenesis of these peptides has been proposed to take place (Kakio et al., 2002; Harris, 2008). Conversely, other research has demonstrated that cholesterol decreases the Aβ-induced changes in structure and morphology of lipid rafts, hindering β-sheet formation in membranes, and thereby reducing peptide insertion, aggregation, and cytotoxicity (Arispe and Doh, 2002; Curtain et al., 2003; Qiu et al., 2011).

The effect of Aβ on cholesterol metabolism has also been investigated: Aβ, especially the oligomeric rather than the monomeric form, may alter the intracellular trafficking and homeostasis of cholesterol, by promoting the release from the cells of cholesterol and other lipids, in the form of Aβ-lipid complexes (Michikawa et al., 2001). It has also been reported that Aβ fibrils down-regulate cholesterol metabolism in cultured neurons (Gong et al., 2002). Additionally, the intracellular domain of APP, which is released upon γ-secretase cleavage of APP, may act as a transcriptional suppressor of LRP1, leading to the down-regulation of cellular cholesterol uptake (Liu et al., 2007) and synaptic failure (Koudinov and Koudinova, 2005), as well as enhancement of tau phosphorylation (Fan et al., 2001). It has also been shown that extracellular cholesterol accumulates in the senile plaques of AD patients, and in transgenic mice expressing the Swedish Alzheimer mutation APP751, by binding to aggregated Aβ (Mori et al., 2001).

Given the above considerations, it appears that cholesterol distribution and trafficking within brain cells, rather than the total amount of cholesterol in the neurons, play key roles in APP processing and in the amyloid cascade during AD progression. Conversely, Aβ may affect cellular cholesterol dynamics, such as transport, distribution, and binding, which in turn have a variety of effects on AD-related pathologic changes leading to neurodegeneration.

A number of epidemiological studies also suggest a positive correlation between hypercholesterolemia and susceptibility to AD, in particular in individuals with the *ApoEε4* genotype, which influences cholesterol metabolism, although this relationship is still the subject of considerable controversy (Shobab et al., 2005; Panza et al., 2006; Jenner et al., 2010; Wood et al., 2014; Luckhoff et al., 2015).

Elevated dietary cholesterol has been reported to increase senile plaque formation in numerous animal models. In double transgenic (APP/PS) mice, consumption of a high-cholesterol

diet (7 weeks) elevated Aβ deposition in the CNS (Refolo et al., 2000). In addition, in transgenic mice, a typical Western diet (1% cholesterol), increased Aβ accumulation and plaque burden, particularly in the dentate gyrus of the hippocampus (Hooijmans et al., 2007). APP23 mice fed a high-fat/high-cholesterol diet for 4 months showed the AD phenotype. This resulted in significantly worse memory deficits than in the same mice fed with a normal diet (Fitz et al., 2010). Furthermore, studies using New Zealand white rabbits have demonstrated that a diet inducing hypercholesterolemia doubles the Aβ concentration in the hippocampal cortex (Sparks et al., 2000). In addition to increasing Aβ, cholesterol-enriched diets increased tau phosphorylation and oxidative stress in rabbit brains (Ghribi et al., 2006; Jaya Prasanthi et al., 2008). It has also been shown that cholesterol colocalizes with fibrillar Aβ in the amyloid plaques of transgenic mice (Burns et al., 2003b). Conversely, a study on guinea pigs showed that lowering total cholesterol profile by administering statins decreased cerebral Aβ production and accumulation (Fassbender et al., 2001).

Although systemic lipoprotein carrying cholesterol cannot cross the BBB, oxysterols, formed during oxidative stress, can cross the BBB, partly because they may have damaging effects on the BBB's integrity and function (Dias et al., 2014). This consideration supports the idea that oxysterols are the link between hypercholesterolemia and AD.

The Involvement of Oxysterols in AD Pathogenesis

To maintain cholesterol homeostasis in the brain, and because the brain cannot degrade cholesterol, there must be a mechanism to eliminate excess cholesterol, transporting it through the BBB into the systemic circulation, thus preventing its accumulation. The most important such mechanism operates via the conversion of cholesterol to oxysterols. Cholesterol is converted into the relatively polar oxysterol 24-OH, which is produced in the brain almost exclusively by cholesterol 24-hydroxylase (CYP46A1) expressed in neurons, and which, unlike cholesterol itself, diffuses across the BBB into the systemic circulation. To a lesser extent, cholesterol is also converted into 27-OH in the brain by cholesterol 27-hydroxylase (CYP27A1), and then into 7α-hydroxy-3-oxo-4-cholestenoic acid (7-OH-4-C) by the enzyme CYP7B; crossing the BBB, 7-OH-4-C reaches the liver where it is eliminated (Björkhem, 2006; Meaney et al., 2007; Björkhem et al., 2009). However, most 27-OH has been found to flow from the systemic circulation into the brain, since it can cross the BBB; here it acts as an important link between extracerebral and intracerebral pools of cholesterol, and may contribute to the negative effects of hypercholesterolemia in the brain (Heverin et al., 2005; Björkhem, 2006; Sharma et al., 2008). A further oxysterol, 7β-hydroxycholesterol (7β-OH), derives from cholesterol oxidation in the brain, following its interaction with APP and Aβ (Nelson and Alkon, 2005). In addition to those oxysterols, others including 7α-hydroxycholesterol (7α-OH), 4β-hydroxycholestrerol (4β-OH), 5α, 6α- and 5β, 6β-epoxicholesterol (α- and β-EPOX) and 7-ketocholesterol (7-K), have recently been identified post-mortem in human AD brains, and their concentrations compared across the disease states (Hascalovici et al., 2009). Of note, the study authors observed that the levels of potentially amyloidogenic sterol species derived from the auto-oxidation of cholesterol, like β-EPOX, were higher at the mild cognitive impairment disease stage. Oxysterols have also been identified in mouse brains and, in addition to the above cited cholesterol derivatives, consistent levels of 25-hydroxycholesterol (25-OH) were found (Ahonen et al., 2014). Increased levels of 24-OH, 7-K, and β-EPOX have also been detected in areas of the rat hippocampus undergoing gliosis and neuroinflammation, after excitotoxic injury (He et al., 2006; Kim et al., 2010). Further, because oxysterols can cross the BBB, the flux of more than 20 cholesterol metabolites between brain and circulation has very recently been verified in 20 patients. Differences in concentrations, between jugular and forearm veins, of 18 oxysterols, 5 cholestenoic acids and 3 cholenoic acids were measured. The study reported that 24-OH and 7-OH-4-C, of enzymatic origin, but also 6-oxo-5α-hydroxycholesterol, 7β-OH, 7-K, which are formed from cholesterol by ROS, are exported from the brain, while 27-OH is imported into brain (Iuliano et al., 2015). Of these cholesterol metabolites, that exported in the largest quantities is 24-OH. Two other cholesterol metabolites, 7α, 25-dihydroxycholest-4-en-3-one and 7α, (25R)26-hydroxycholest-4-en-3-one, were reported to be exported from the brain (Crick et al., 2015b). Oxysterols and cholestenoic acids have also been identified and quantified in mouse cerebrospinal fluid (CSF), and the findings compared with concentrations of the same metabolites found in the plasma, in order to clarify cholesterol metabolism. Concentrations of oxysterols were lower in the CSF than in the plasma, but 7α, 24-dihydroxycholesterol and 7α, 24-dihydroxycholest-4-en-3-one, both of enzymatic origin, were only identified in the CSF (Crick et al., 2015a). These data clearly demonstrate that there are several routes by which cholesterol metabolites may be exported or imported from the brain (**Figure 2**).

The idea has therefore gained ground that, owing to their ability, unlike cholesterol, to cross the BBB, oxysterols might be the missing link between altered brain cholesterol metabolism and AD pathogenesis, as well as between hypercholesterolemia and AD (Gamba et al., 2012). Although this means that the brain can eliminate excess amounts of oxysterols, it could conversely allow toxic amounts of these compounds, present in the bloodstream, to accumulate in the brain, as in the case of 27-OH. The key role of oxysterols in AD pathogenesis has been strongly supported by the last decade's research, pointing to the involvement of these oxysterols in the amyloid cascade.

To date, although contradictory results could obviously arise from future findings on the other cholesterol metabolites found in the brain, the oxysterols most widely considered to be potentially implicated in the pathogenesis of AD are 24-OH and 27-OH, both of enzymatic origin (Iuliano, 2011; Jeitner et al., 2011; Leoni and Caccia, 2011; Gamba et al., 2012; Hughes et al., 2013; Marwarha and Ghribi, 2014; Noguchi et al., 2014; **Table 1**).

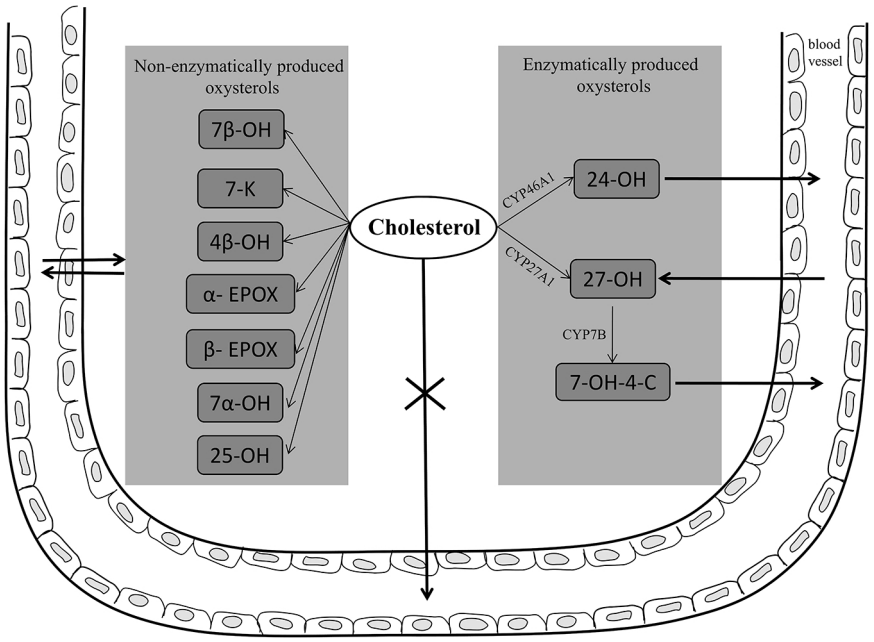

FIGURE 2 | Enzymatically and non-enzymatically produced oxysterols in AD brain and their fluxes across the blood brain barrier (BBB). In neuronal cells, cholesterol is converted into 24-hydroxycholesterol (24-OH) by the enzyme CYP46A1; 24-OH, unlike cholesterol, diffuses across the BBB into the systemic circulation. To a lesser extent, cholesterol is also converted into 27-hydroxycholesterol (27-OH) by the enzyme CYP27A1, and then into 7α-hydroxy-3-oxo-4-cholestenoic acid (7-OH-4-C) by the enzyme CYP7B; crossing the BBB, 7-OH-4-C reaches the liver where it is eliminated. However, most 27-OH flows from the circulation into the brain, since it can cross the BBB. In addition, other oxysterols, such as 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-K), 7α-hydroxycholesterol (7α-OH), 4β-hydroxycholestrerol (4β-OH), 5α, 6α- and 5β, 6β-epoxicholesterol (α- and β-EPOX), and 25-hydroxycholesterol (25-OH), have been found in AD brain deriving from brain cholesterol autoxidation. Potentially these oxysterols, as well as other cholesterol metabolites, can cross the BBB.

TABLE 1 | 24-hydroxycholesterol and 27-hydroxycholesterol levels in Alzheimer's disease patients compared with healthy controls.

Oxysterol	Levels of oxysterol in AD subjects compared with control subjects	Reference
24-hydroxycholesterol	↓ Brain levels	Heverin et al. (2004)
	↑ CSF levels	Papassotiropoulos et al. (2002), Schönknecht et al. (2002) and Leoni et al. (2004)
	↓ Plasma levels	Bretillon et al. (2000) and Kölsch et al. (2004)
	↑ Plasma levels	Lütjohann et al. (2000)
	= Plasma levels	Iuliano et al. (2010)
27-hydroxycholesterol	↑ Brain levels	Heverin et al. (2004) and Shafaati et al. (2011)
	↑ CSF levels	Leoni et al. (2004)
	↓ Plasma levels	Kölsch et al. (2004)
	= Plasma levels	Iuliano et al. (2010)

CSF, cerebrospinal fluid.

Higher levels of 24-OH than in unaffected individuals have been found in the peripheral circulation and CSF of early-stage AD patients, suggesting that cholesterol turnover in the brain increases during the neurodegenerative changes of AD (Lütjohann et al., 2000; Papassotiropoulos et al., 2002; Kölsch et al., 2004). Conversely, plasma levels of 24-OH were lower in patients with later stages of AD than in unaffected individuals, suggesting that the rate of cholesterol transport slows as the disease progresses (Bretillon et al., 2000; Kölsch et al., 2004). These apparently contradictory results may be rationalized by considering that increased plasma levels of 24-OH reflect ongoing neurodegeneration and/or demyelinization, whereas decreased plasma levels in later stages reflect a selective loss of neuronal cells expressing the enzyme CYP46A1 (Björkhem and Meaney, 2004). The decrease of 24-OH in the AD brain may also be the result of an increase in total free cholesterol (likely derived from cell membrane collapse and widespread myelin release) that exceeds the brain's capacity to convert it to 24-OH (Vaya and Schipper, 2007). However, it has been observed that, in glial cells, and especially around senile plaques, there is an ectopic induction of CYP46A1, leading to some 24-OH production, but without compensating for the decrease of that

oxysterol (Bogdanovic et al., 2001; Brown et al., 2004). Another study, however, has found that plasma levels of 24-OH and 27-OH in AD patients are not significantly different from control values (Iuliano et al., 2010). A small fraction of total 24-OH excretion occurs via the CSF, and the 24-OH concentration is increased in the CSF of AD patients, probably reflecting neuronal damage and loss rather then metabolically active neuronal cells (Schönknecht et al., 2002; Leoni et al., 2004).

It has also been reported that, in critical areas of post-mortem AD brains, as well as in aged mice expressing the Swedish Alzheimer mutation APP751, 24-OH levels are decreased and those of 27-OH increased (Heverin et al., 2004). Increased levels of 27-OH were also found in the brains of patients carrying the Swedish APP670/671 mutation (Shafaati et al., 2011). As a consequence of neuron loss, expression of CYP27A1 may also be reduced; however, 27-OH levels remain elevated because CYP27A1 is also expressed in astrocytes and oligodendrocytes, leading to *in situ* generation of 27-OH (Brown et al., 2004). Accumulation of 27-OH in the brain is also due to the increased flux of this oxysterol across the BBB, because of either hypercholesterolemia associated to oxidative stress (Björkhem, 2006), or damaged BBB integrity (Leoni et al., 2003). There is a positive correlation between levels of cholesterol and those of 27-OH in the circulation, and the high flux of 27-OH from the peripheral circulation into the brain suggests that this oxysterol may be the link between hypercholesterolemia and AD. An alternative explanation for the accumulation of 27-OH is reduced activity of CYP7B, the neuronal enzyme responsible for 27-OH metabolism; this reduction arises from the reduced CYP7B expression in the brain of AD patients, because of neuron loss (Yau et al., 2003). Moreover, high CSF levels of 27-OH were found in mild cognitive impairment and AD patients (Leoni et al., 2004).

From these considerations, it is clear that the balance between 24-OH and 27-OH levels is important. Oxysterol homeostasis in the brain is tightly regulated, specific levels being maintained in various brain regions. For example, the 27-OH:24-OH ratio is ∼1:8 in the frontal cortex, 1:5 in the occipital cortex, and 1:10 in the basal ganglia, and the increased ratio of 27-OH to 24-OH in AD brains is consistent with AD pathogenesis. Thus it is likely that reduced levels of 24-OH may accelerate disease progression, and that the increased levels of 27-OH may be insufficient to compensate for this: the shift in balance between the two oxysterols might lead to increased generation and accumulation of Aβ with consequent neurodegeneration (Heverin et al., 2004; Björkhem, 2006; Björkhem et al., 2009). In a retrospective study on cardiovascular patients with evidence of cerebrovascular disease, higher plasma levels of 24-OH and a higher 24-OH/27-OH ratio were found to be associated with the development of incidental cognitive impairment over 8 years of follow-up (Hughes et al., 2012). However, opinions still differ about the involvement of 24-OH and 27-OH in APP processing and Aβ production.

To date, the conversion of cholesterol into 24-OH, by inducing CYP46A1 activity, has been considered to exert a protective action on the brain, mainly by regulating cholesterol homeostasis and favoring the efflux of its excess from the brain to the blood, but also by preventing Aβ generation (Björkhem et al., 2009). Astrocytes are sensitive to levels of 24-OH, which regulates the expression of LXR-responsive genes involved in cholesterol homeostasis (i.e., *ABCA1*, *ABCG1* and *ApoE*) (Abildayeva et al., 2006). Indeed, 24-OH acts as an endogenous ligand of LXR. Conversely, it has been reported that brain accumulation of 27-OH antagonizes the preventive effect of 24-OH on generation of Aβ and that it is potentially toxic (Shafaati et al., 2011). Since the flux of 27-OH across the BBB increases under conditions of hypercholesterolemia (Björkhem, 2006), or in the case of increased BBB permeability (Leoni et al., 2003), the inhibitory effect of 24-OH on Aβ generation would consequently be reduced.

Studies on human neuroblastoma cells and on brain tissues have somewhat clarified the different effects of 24-OH and 27-OH on APP levels and processing: 24-OH may favor the non-amyloidogenic pathway, with consequent inhibition of Aβ formation, whereas 27-OH is thought to stimulate the amyloidogenic pathway, with production of Aβ as well as tau hyperphosphorylation (Bu, 2009; Prasanthi et al., 2009; Marwarha et al., 2010). However, the mechanisms underlying their different effects are still unclear.

In human SH-SY5Y neuroblastoma cells, 24-OH appears to exert a unique modulatory effect on APP processing: it directly increases α-secretase activity, as well as elevating the α/β-secretase activity ratio; conversely, 27-OH enhances the generation of Aβ (Famer et al., 2007). *In vitro* experiments suggest that 24-OH reduces Aβ production, by down-regulating APP trafficking via enhancement of the complex formation of APP, also up-regulating glucose-regulated protein 78 in the endoplasmic reticulum. The inhibitory effect of 24-OH was reduced in glucose-regulated protein 78 knockdowned cells (Urano et al., 2013). In rat primary cortical neurons, both 24-OH and 27-OH were found to be inhibitors of Aβ secretion, 24-OH being approximately 1000 times more potent than 27-OH (Brown et al., 2004). SH-SY5Y cells incubated with 27-OH release higher levels of $A\beta_{1-42}$ and APP as well as of BACE1. Conversely, cells incubated with 24-OH do not release increased $A\beta_{1-42}$ levels, and are associated with increased levels of sAPPα, suggesting that 24-OH favors APP processing via the non-amyloidogenic pathway (Prasanthi et al., 2009). Another study, on hippocampal slices from adult rabbits, found that 27-OH increases Aβ accumulation by reducing levels of insulin-like growth factor 1, a neurotrophic factor that promotes neurogenesis and has a neuroprotective effect (Sharma et al., 2008). This oxysterol has also been found to increase both BACE1 and Aβ levels in retinal pigment epithelial cells (Dasari et al., 2010). In neuronal SK-N-BE cells, 24-OH and 27-OH have both been shown to enhance expression and activity of the β-secretase of the amyloidogenic pathway of APP processing, leading to increased Aβ generation and accumulation in those cells (Gamba et al., 2014; **Table 2**).

24-OH has also been reported to be neurotoxic, but this effect may depend on its concentration. It has been demonstrated that high concentrations of 24-OH (25–50 μM) caused cell death when added to undifferentiated or differentiated SH-SY5Y cells: the effect was mediated by increased generation of

TABLE 2 | Effects of 24-hydroxycholesterol and 27-hydroxycholesterol on the amyloidogenic pathway.

Oxysterol	Dosage	Effects	Experimental model	Reference
24-hydroxycholesterol	*C* = 10 μM	↓ Aβ production	Primary culture of rat cortical neurons	Brown et al. (2004)
	C = 5 μM	↑ α-secretase activity ↓ β-secretase activity	Undifferentiated human neuroblastoma cell line SH-SY5Y	Famer et al. (2007)
	C = 10 μM	↑ α-secretase activity no effect on APP, BACE1 and Aβ level	Undifferentiated human neuroblastoma cell line SH-SY5Y	Prasanthi et al. (2009)
	C = 1–10 μM	↑ APP level ↓ Aβ production no effect on β-secretase activity	Undifferentiated SH-SY5Y cells and Chinese hamster ovary (CHO) cells	Urano et al. (2013)
	C = 1 μM	↑ APP level and Aβ production ↑ α-secretase level ↑ β-secretase level and activity	Differentiated human neuroblastoma cell line SK-N-BE	Gamba et al. (2014)
27-hydroxycholesterol	*C* = 1–15 μM	↓ Aβ production	Primary culture of rat cortical neurons	Brown et al. (2004)
	C = 5 μM	No effect on α- and β-secretase activity	Undifferentiated human neuroblastoma cell line SH-SY5Y	Famer et al. (2007)
	C = 10 μM	↑ Aβ production ↑ APP and BACE1 level	Undifferentiated human neuroblastoma cell line SH-SY5Y	Prasanthi et al. (2009)
	C = 10–25 μM	↑ Aβ production ↑ BACE1 level	Retinal pigmented epithelial cells ARPE-19	Dasari et al. (2010)
	C = 5 μM	↑ Aβ production ↑ BACE1 level and activity	Undifferentiated human neuroblastoma cell line SH-SY5Y	Marwarha et al. (2013)
	C = 1 μM	↑ APP level and Aβ production ↑ α-secretase level ↑ β- and γ-secretase level and activity	Differentiated human neuroblastoma cell line SK-N-BE	Gamba et al. (2014)

Aβ, amyloid β; APP, amyloid precursor protein; BACE1, beta-site amyloid precursor protein cleaving enzyme 1.

free radicals (Kölsch et al., 2001). High concentrations of 24-OH (50 μM) also induce necroptosis, a form of programmed necrosis in neuronal SH-SY5Y cells (Yamanaka et al., 2011). In contrast, pretreatment of human neuroblastoma SH-SY5Y cells with sub-lethal concentrations of 24-OH induces adaptive responses, and protects the cells against subsequent cytotoxic stress induced by 7-K treatment, via transcriptional activation of the LXR signaling pathway. The cytoprotective effects of 24-OH disappeared in LXRβ-knockdowned cells, suggesting that this nuclear receptor may play a key role in the 24-OH-induced adaptive response. Adaptive responses are also induced by other oxysterols, such as 25-OH and 27-OH, both ligands of LXR, similarly to 24-OH (Okabe et al., 2013). However, in our recent studies, a very low concentration (1 μM) of 24-OH was found to markedly potentiate both the apoptotic and the necrogenic effects exerted by the $A\beta_{1-42}$ peptide, on two human differentiated neuronal cell lines (SK-N-BE and NT-2) (Gamba et al., 2011), but also on human dental pulp progenitor cells differentiated into neuron-like cells (Testa et al., 2012). 24-OH appeared to interact with $A\beta_{1-42}$ by strongly increasing intracellular ROS steady-state levels, an action not exerted by either 27-OH or 7β-OH (Gamba et al., 2011). This evidence supports the opinion that Aβ must form oligomers in order to induce neurotoxicity, and that the latter process is probably enhanced by redox imbalance. Additionally, 50 μM 24-OH has been shown to enhance the neurotoxic effect of the $A\beta_{1-42}$ peptide in the human differentiated neuroblastoma cell line MSN, as well as augmenting ROS generation (Ferrera et al., 2008). Although in many of the various *in vitro* tests performed in our laboratory, 27-OH (1 μM) did not display neurotoxicity, in terms of necrosis and apoptosis (Gamba et al., 2011), conversely, the toxicity of 27-OH (5–20 μM) has been demonstrated in astrocyte cells (C6 cells). This oxysterol increased ROS levels and decreased antioxidant defense system levels, with consequent decrease of cell viability. In addition, 27-OH down-regulated the expression of the nuclear factor E2-related factor 2 signaling pathway (Ma et al., 2015).

It is known that hypertension is a risk factor for AD, and that angiotensin converting enzyme activity is increased in AD brains. In this connection, it has been suggested that 27-OH might up-regulate the renin-angiotensin system in AD brains. A positive correlation between angiotensin converting enzyme activity in the CSF, and both plasma and CSF levels of 27-OH, has been shown, as well as an increased production of angiotensinogen in rat primary neurons, astrocytes, and human neuroblastoma cells treated with 27-OH (Mateos et al., 2011).

Moreover, 7β-OH has been found to be neurotoxic at very low concentrations on cultured rat hippocampal neuronal cells, and may therefore contribute to neurodegeneration in AD brains. In the same study, it has also been shown that Aβ can oxidize cholesterol to form 7β-OH in a highly efficient mechanism and more actively than APP. Oxidation of cholesterol was accompanied by hydrogen peroxide production, suggesting that Aβ could contribute to the oxidative damage observed in AD (Nelson and Alkon, 2005). 7β-OH causes re-arrangement

of the liquid-ordered phase which results in the formation of lipid rafts (Wang et al., 2008; Mitomo et al., 2009); it is also a potent inhibitor of α protein kinase C, an enzyme critical for memory consolidation and synaptic plasticity that is implicated in AD (Nelson and Alkon, 2005). Another oxysterol that might derive from the autoxidation of cellular cholesterol released during neurodegeneration is 7α-OH, which appeared to be responsible for SH-SY5Y cell death (Kölsch et al., 2000); a further possibility is 7-K. 7-K has been shown to enhance mitochondrial dysfunction in the neuronal PC12 cell line, leading to cell death (Kim et al., 2006; Kim and Lee, 2010; Jang and Lee, 2011). It has also been reported that incorporation of 7-K to lipid raft domains of plasma membranes triggers apoptotic signaling; α-tochopherol (vitamin E) reduces the cytotoxicity of 7-K by inhibiting its distribution to the lipid raft domains (Berthier et al., 2004; Royer et al., 2009). It has recently been suggested that 25-OH is an important regulator of cholesterol metabolism, as well as of humoral immunity (Diczfalusy, 2013; Waltl et al., 2013). Of note, it has also been hypothesized that Aβ deposition is not a central event in AD, but rather is subservient to 25-OH. Confirming this hypothesis, it has been observed that, in a large cohort of AD patients, specific *25-hydroxylase* haplotypes were associated with a complete absence of Aβ deposits in the brain, despite all other aspects of AD pathology being present (Papassotiropoulos et al., 2005). This suggests that neuroinflammation and 25-hydroxylase activation precede Aβ generation in the sequence of events leading to the disease. An interesting study on different immortalized, tumoral and normal cells of the CNS has found that oxysterols oxidized at C4, such as 4α-OH and 4β-OH, have no effect on cell viability and almost no effect on cell growth; conversely, oxysterols oxidized at C7, such as 7-K, 7α-OH, and 7β-OH, inhibit cell growth and decrease viability through their cytotoxic activity. These data suggest that 4α-OH and 4β-OH, the only oxysterols identified as having cytostatic properties, may be of some interest for attempts to counteract cell proliferation (Nury et al., 2013).

Oxysterols have also been shown to modify specific sites of the Aβ peptide thus enhancing Aβ aggregation and its neurotoxicity. Following Aβ modification at Lys-16, peptide aggregates were formed faster than in the case of modification at Lys-28 or at Asp-1 (Usui et al., 2009).

Further, evidence has emerged that Aβ has predominant cholesterol oxidase activity, particularly in the presence of divalent cations such as Cu^{2+}. Significantly elevated levels of 4-cholesten-3-one were reported in brains of Aβ transgenic mice and in brain tissue of AD patients (Puglielli et al., 2005; Yoshimoto et al., 2005).

The interaction of Aβ with cell membranes is the crucial event in AD pathogenesis. Of note, there is less evidence to date on the negative effects of oxysterols on Aβ binding to the cell membranes. Because the orientation of oxysterols in the cell membrane differs from that of cholesterol, they are less able to condense lipids, thus modifying some physical properties of membrane, including raft domains; it has thus been suggested that oxysterols may facilitate Aβ interaction with cell membranes. The effects of 7-K and 7β-OH on enhancing Aβ insertion into the lipid bilayer, by decreasing intermolecular cohesive interaction, have been demonstrated (Kim and Frangos, 2008). Using a model membrane, it was shown that 25-OH and 7-K render the membrane more sensitive to Aβ, in contrast to the role played by cholesterol, which inhibits Aβ's interaction with membranes. 7-K facilitated Aβ's localization in the membrane, while 25-OH stimulated the peptide's insertion but lead to membrane modification. In addition, the higher potential of $Aβ_{1–42}$, compared to $Aβ_{1–40}$, to interact with the membrane has also been demonstrated (Phan et al., 2013). Further, it is hypothesized that increased oxysterol concentrations, mainly of 7-K, but also of 24-OH and β-EPOX, may enhance exocytosis and neurotransmitter release in damaged areas of the brain, thereby aggravating neuronal excitotoxicity (Ma et al., 2010). Further, in our study we observed that 24-OH, 27-OH, and 7β-OH markedly enhanced the binding of $Aβ_{1–42}$ on membranes of human differentiated neuronal cell lines (SK-N-BE and NT-2), by up-regulating CD36 and β1-integrin receptors (Gamba et al., 2011), two components of the multireceptor complex CD36/β1-integrin/CD47, through which Aβ peptide binds to cell membranes (Verdier et al., 2004; Yu and Ye, 2015). This event might favor the accumulation of the toxic $Aβ_{1–42}$ peptide into neurons.

Although oxysterols have been analyzed for their involvement in neurotoxicity and Aβ production during AD progression, their role as natural ligands for LXR is now emerging (e.g., 24-OH, 27-OH, 22-OH, 25-OH, 4β-OH, and 7α-OH) (Vaya and Schipper, 2007). Indeed, astrocytes are sensitive to 24-OH-mediated up-regulation of ApoE, a LXR-target gene involved in cholesterol efflux (Abildayeva et al., 2006). Moreover, it has been reported that 27-OH prevents Aβ generation from primary human neurons, not by modulating α-, β-, or γ-secretase, but rather by overexpressing LXR-responsive genes (*ABCA1*, *ABCG1* and *ApoE*) (Kim et al., 2009b). Moreover, incubation of primary brain cells with 22-OH significantly reduced Aβ secretion in a dose-dependent manner, while ABCA1 expression and cholesterol efflux were induced (Koldamova et al., 2003).

Recent *in vitro* evidence also suggests that 24-OH and 27-OH might contribute to decreasing the influx of Aβ peptide into the brain across the BBB, increasing expression of the ABCB1 transporter in brain capillary endothelial cells, resulting in protection from peripheral Aβ entry (Saint-Pol et al., 2013). Of note, ABCB1 has never been described as an LXR target gene, and other nuclear receptors might control its transcription. Conversely, treatment of brain pericytes with 24-OH up-regulated ABCA1 expression that was correlated with an increase of cholesterol efflux, whereas 24-OH treatment did not reduce the pericytes' ability to accumulate Aβ in the cells (Saint-Pol et al., 2012). The clearance of Aβ might also be mediated through microglia-induced phagocytosis of Aβ, which depends on LXR activation (Terwel et al., 2011).

Of note, LXR activation not only regulates cholesterol homeostasis, and Aβ peptide transport and clearance, but also neuroinflammation. Studies have shown that LXR activation inhibits inflammatory gene expression, pointing to the ability of LXRs to inactivate promoters of pro-inflammatory genes

(Wang et al., 2002; Cao et al., 2007; Zelcer et al., 2007; Sodhi and Singh, 2013; Steffensen et al., 2013). Moreover, LXR activation may prevent neuroinflammation, by indirectly down-regulating TLR target genes. However, although LXR-activating oxysterols might reduce membrane cholesterol content and inflammation, they may also activate opposing pathways, and induce inflammation independently of LXRs. In our very recent study, we observed that 27-OH, 24-OH, and 7β-OH enhanced inflammatory molecule expression in human neuroblastoma SH-SY5Y cells via TLR4/cyclooxygenase-2/membrane bound prostaglandin E synthase; this clearly indicates that oxysterols may promote neuroinflammation in AD (Testa et al., 2014).

Although it can be assumed that oxysterols may increase the activation of microglia promoting their phagocytosis, there is less evidence to date on their effects on microglial phagocytosis during neuroninflammation. The phagocytosis of fibrils and large aggregates of Aβ by microglia is an important neuroprotective mechanism for Aβ peptide clearance (D'Andrea et al., 2004; Colton and Wilcock, 2010) but, in later stages of AD, the increased inflammatory molecule release makes the microglia phagocytically inactive leading to neuronal death (Hickman et al., 2008; Krabbe et al., 2013). Among the receptors promoting Aβ phagocytosis and clearance by microglia, the CD36 scavenger receptor appears to be involved and its increased expression may be crucial in preventing AD (Verdier et al., 2004; Yu and Ye, 2015): CD36 initiates a signaling cascade that promotes microglial activation and recruitment to β-amyloid deposits in the brain (Stuart et al., 2007). Concernig sterols, it has been shown that cholesterol (20 μM) and α-EPOX (20 μM) do not interfere with CD36 membrane distribution but both compounds were found to up-regulate the total CD36 levels in the mouse microglial cell line BV-2 potentiating phagocytosis in LPS-stimulated cells (Račková, 2013). Moreover, treatment with methyl-β-cyclodextrin, a reagent able to remove cholesterol from cell membranes, inhibited phagocytosis in LPS-activated microglia, indirectly supporting the potential role of sterols in phagocytosis (Churchward and Todd, 2014).

Conclusion

This review has pointed up the vicious circle connecting oxidative stress and inflammation in AD. Alongside oxidative stress and neuroinflammation, altered cholesterol metabolism in the brain and hypercholesterolemia also significantly contribute to AD pathogenesis. Thanks to consistent research evidence, it is now believed that oxidized cholesterol is the driving force behind the development of AD and that oxysterols are the link connecting altered cholesterol metabolism and hypercholesterolemia to this neurodegenerative disease. Oxysterols play a fundamental role, by enhancing inflammation, Aβ generation and accumulation, and neuron death.

The involvement of oxysterols in AD pathogenesis, and the analysis of such products in the plasma and CSF, may contribute to clarifying the role of cholesterol metabolism in AD; ultimately, it may be helpful in developing therapeutic strategies to prevent or slow AD pathogenesis.

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Brain metabolic stress and neuroinflammation at the basis of cognitive impairment in Alzheimer's disease

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Brain metabolic dysfunction is known to influence brain activity in several neurological disorders, including Alzheimer's disease (AD). In fact, deregulation of neuronal metabolism has been postulated to play a key role leading to the clinical outcomes observed in AD. Besides deficits in glucose utilization in AD patients, recent evidence has implicated neuroinflammation and endoplasmic reticulum (ER) stress as components of a novel form of brain metabolic stress that develop in AD and other neurological disorders. Here we review findings supporting this novel paradigm and further discuss how these mechanisms seem to participate in synapse and cognitive impairments that are germane to AD. These deleterious processes resemble pathways that act in peripheral tissues leading to insulin resistance and glucose intolerance, in an intriguing molecular connection linking AD to diabetes. The discovery of detailed mechanisms leading to neuronal metabolic stress may be a key step that will allow the understanding how cognitive impairment develops in AD, thereby offering new avenues for effective disease prevention and therapeutic targeting.

Keywords: Alzheimer's disease, amyloid-β oligomers, endoplasmic reticulum stress, inflammation, metabolic stress

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Introduction

Incidence of Alzheimer's disease (AD) will greatly increase as world population ages (Prince et al., 2013) and changes in lifestyle observed in recent decades seem to be major contributors to such increased prevalence (Mattson, 2012; De Felice, 2013). Likewise, common diseases of modern adulthood, including obesity and diabetes mellitus, have been often regarded as AD risk factors (De Felice, 2013). Pioneering epidemiological studies connecting AD to diabetes initiated in the 1990s (Ott et al., 1996, 1999; Kalmijin et al., 1997) and were followed by several reports providing both clinical and experimental evidence into how these two disorders may course together (de la Monte, 2009; Matsuzaki et al., 2010; Crane et al., 2013; De Felice, 2013; De Felice et al., 2014).

Metabolic derangements, including inflammation, insulin resistance and endoplasmic reticulum (ER) stress, are known to underlie glucose intolerance and type 2 diabetes mellitus (T2DM) in peripheral tissues (Hotamisligil et al., 1995, 1996; Ozcan et al., 2004, 2006; Hotamisligil, 2006). A similar scenario has been recently described in the brains of patients that suffer from neurodegenerative disorders, such as AD. Neuropathology investigations have revealed that AD brains present several markers of insulin resistance, inflammation and ER stress (Hoozemans et al., 2005; Steen et al., 2005; Moloney et al., 2010; Bomfim et al., 2012; Talbot et al., 2012; O'Neill, 2013;

De Felice et al., 2014). In the following sections, we review current evidence indicating that a newly defined form of metabolic stress leads the path to cognitive decline in AD. The understanding of molecular mechanisms driving AD pathogenesis may shed new light on novel targets for drug development and offer strategies for disease prevention.

AD Pathogenesis

AD pathophysiology includes neuroinflammation, oxidative and ER stress, synapse loss and degeneration of specific neuronal populations (Selkoe, 2002; Ferreira and Klein, 2011; Mucke and Selkoe, 2012). Amyloid-β peptide (Aβ) is the main component of senile plaques that accumulate in AD brains (Masters et al., 1985), and substantial evidence indicates that Aβ is causally involved in AD (Mucke and Selkoe, 2012). Consolidated knowledge has established that soluble Aβ oligomers (AβOs; Lambert et al., 1998), and not necessarily the insoluble amyloid fibrils detected in senile plaques, promote direct damage to synapses, besides stimulating inflammatory response and cellular stress (Ferreira and Klein, 2011; Viola and Klein, 2015). These findings prompt AβOs, which are increased in AD brains (Gong et al., 2003; Xia et al., 2009), to be considered neurotoxins responsible for synapse and memory loss in AD early stages.

Very recent data has demonstrated that AβO actions stimulate pro-inflammatory mechanisms to impair neuronal insulin signaling and to trigger stress kinase activation, resulting in synapse and memory impairments in AD models (Bomfim et al., 2012; Lourenco et al., 2013; Ma et al., 2013). These events are quite similar to those acting in peripheral tissues to impair metabolism in diabetes and obesity (De Felice and Ferreira, 2014), in line with the idea that a form of metabolic stress develops in AD brains (Kapogiannis and Mattson, 2011; Yoon et al., 2012; De Felice and Ferreira, 2014). Such findings may impact translational research, as treating brain metabolic dysfunction might be a key strategy to fight neurological disorders.

Brain Metabolic Stress Mechanisms in AD

In peripheral tissues, prolonged inflammatory cascades lead to the activation of multiple cellular stress mechanisms that ultimately impair cell function and body metabolism (Hotamisligil, 2006; Gregor and Hotamisligil, 2011). In AD, evidence arising from *in vitro*, *in vivo* and neuropathology studies supports that such events occur throughout disease development and are linked to AβO neurotoxicity. Oligomers promote neuronal stress by instigating abnormal elevations in levels of tumor necrosis factor α (TNF-α) and reactive oxygen species (ROS), as well as activation in JNK/PKR signaling and increased eIF2α phosphorylation (eIF2α-P) levels in AD models (De Felice et al., 2007, 2014; Ma et al., 2009, 2013; Bomfim et al., 2012; Lourenco et al., 2013). In this context, pro-inflammatory signals appear to be directly responsible for defective insulin signaling and stress-mediated synapse loss caused by AβOs in neurons (Bomfim et al., 2012; Lourenco et al., 2013). This has led to a concept in which AβOs build up in pre-AD brains to cause inflammation (e.g., gliosis and cytokine production) and neuronal metabolic stress, ultimately leading to synaptic dysfunction and behavioral alterations. We next detail some of the mechanisms recently implicated in AD pathogenesis.

Unfolded Protein Response

Unfolded Protein Response (UPR) is defined as a collection of signaling pathways that respond to ER stress due to accumulation of misfolded proteins and/or impaired homeostasis. ER membrane sensors activate three signaling axes (ATF6α, IRE-1α/XBP-1s and PERK/eIF2α-P) to instigate transcriptional and translational alterations aimed at restoring cell homeostasis (Lai et al., 2007; Hetz et al., 2013). UPR signaling attenuates global translation and favors the synthesis of select transcription factors, such as ATF4, CHOP and Nrf2 (Buffington et al., 2014; Hetz and Mollereau, 2014). Under continued stress, however, these pathways may promote cell damage and death. This hormetic response pattern is thus critical to determine cell fate in such conditions (Mattson, 2008; Hetz, 2012).

Evidence for canonical UPR activation has been found in AD neurons (Hoozemans et al., 2005, 2009; Yoon et al., 2012) and in AD mouse models (Yoon et al., 2012; Ma et al., 2013). In accordance, AβOs trigger UPR in hippocampal neurons *in vitro* and *in vivo* (Chafekar et al., 2007; Casas-Tinto et al., 2011; Lourenco et al., 2013; Barbero-Camps et al., 2014), and experimental induction of ER stress leads to neuronal metabolic stress (Yoon et al., 2012), tau phosphorylation (Bose et al., 2011; van der Harg et al., 2014), stress kinase activation (Bose et al., 2011; Paquet et al., 2011) and cognitive impairment in mice (Lourenco et al., 2013). Further, alleviating ER stress with 4-phenylbutyrate, a chemical chaperone, promotes cognitive benefits in AD mouse models (Ricobaraza et al., 2009, 2010; Wiley et al., 2011; Lourenco et al., 2013).

Substantial recent evidence has proposed that UPR activation is a common feature of different neurodegenerative diseases, as deleterious impacts of UPR branches were reported in AD (Lourenco et al., 2013; Ma et al., 2013; Barbero-Camps et al., 2014; van der Harg et al., 2014), Parkinson's (Bellucci et al., 2011; Colla et al., 2012), Huntington (Lajole and Snapp, 2011), amyotrophic lateral sclerosis (ALS; Hetz et al., 2009; Kim et al., 2013) and prion diseases (Moreno et al., 2012). Correcting UPR activation further appears to be effective in preclinical models of prion infection (Moreno et al., 2012, 2013; Halliday et al., 2015) and ALS (Hetz et al., 2009; Kim et al., 2013), in addition to AD models (Ricobaraza et al., 2009, 2010; Lourenco et al., 2013; Ma et al., 2013). Therefore, it is likely that aberrant UPR signaling mediates brain dysfunction in a variety of neurological conditions (**Figure 1**).

eIF2α-P and Translational Repression

Under cellular stress, translational repression can be mediated by increased eIF2α phosphorylation (eIF2α-P), a regulatory factor essential for translation initiation in eukaryotes (Raven and Koromilas, 2008). PERK-mediated eIF2α-P is the main UPR branch leading to general protein synthesis repression and

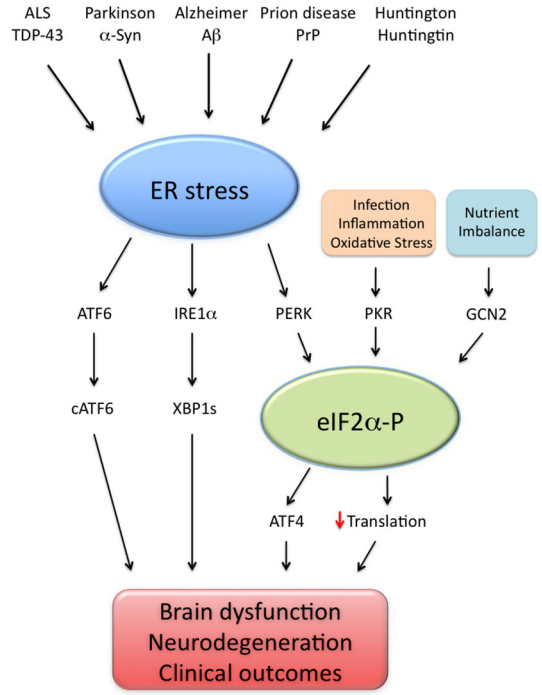

FIGURE 1 | Endoplasmic reticulum (ER) stress as a common denominator of neurodegenerative diseases. Brain ER stress is caused by several disease-associated stressors, including amyloid-β (Aβ; Alzheimer disease), α-synuclein (α-syn; Parkinson disease), prion protein (PrP; prion-related diseases), mutated huntingtin (Huntington and poly-Q diseases) and TAR DNA-binding protein of 43 kDa (TDP-43; amyotrophic lateral sclerosis, ALS). In such disorders, abnormal ER stress leads to the activation of three signaling pathways (ATF6; IRE1α/XBP1 s and PERK/eIF2α-P) collectively termed unfolded protein response (UPR). In parallel, events of infection, inflammation, nutrient deprivation and oxidative stress activate additional eIF2α kinases (namely PKR and GCN2), leading to abnormally high eIF2α-P levels, increased ATF4 production and impaired translation. Excessive UPR activity, eIF2α-P signaling and their downstream effectors impair cell function and may result in brain dysfunction and neurodegeneration, possibly explaining the clinical outcomes observed in neurodegenerative conditions.

facilitation of select mRNA translation (Buffington et al., 2014). Both PERK and eIF2α-P appear to be elevated in AD brains (Chang et al., 2002b; Yoon et al., 2012; Ma et al., 2013) and are induced by Aβ aggregates in neurons (Lee et al., 2010). Increased eIF2α-P has been further verified in other AD mouse models (Segev et al., 2012; Devi and Ohno, 2014).

Two other eIF2α kinases, namely the stress kinase PKR and the nutrient sensor GCN2, are enriched in the brain and have been reported to increase neuronal eIF2α-P (Costa-Mattioli et al., 2005; Lourenco et al., 2013; Roffé et al., 2013; Hetz and Mollereau, 2014), and thus emerge as candidates to explain increased eIF2α-P in AD.

Interestingly, deletion of either PERK or GCN2 in the brains of APP/PS1 mice decreases eIF2α-P levels, rescuing synapse plasticity and cognition (Ma et al., 2013). AβOs increase eIF2α-P through TNF-α-dependent PKR activation, thereby promoting synapse loss in hippocampal neurons and cognitive impairment in mice (Paquet et al., 2011; Lourenco et al., 2013). Providing clinical relevance to the findings observed in experimental models, PKR was found to be abnormally active in AD brains (Chang et al., 2002a; Paquet et al., 2011; Mouton-Liger et al., 2012b). Therefore, it is likely that PERK, GCN2, and PKR lead to increased eIF2α-P levels in AD.

Increased eIF2α-P levels also facilitate the translation of a small fraction of mRNAs (Buffington et al., 2014), among which is activating transcription factor 4 (ATF4), a protein linked to oxidative stress, enhanced γ-secretase activity and neuronal dysfunction when abnormally elevated (Mitsuda et al., 2007; Lange et al., 2008). ATF4 signaling further counteracts CREB1 pro-memory actions in mice (Costa-Mattioli et al., 2005; Rajasethupathy et al., 2012).

Recent findings demonstrated that ATF4 levels are increased in AD brains (Yoon et al., 2012; Baleriola et al., 2014) and in AD animal models (Ma et al., 2013; Devi and Ohno, 2014). Furthermore, soluble Aβ species appear to locally stimulate axonal ATF4 translation to propagate a neurodegenerative message in mice (Baleriola et al., 2014). Hence, eIF2α-P/ATF4 signaling has the potential to explain, at least in part, how disease progresses from defined brain regions in the beginning to a widespread forebrain dysfunction at later stages.

Translational repression instigated by eIF2α-P may be harmful to cognition, given that normal protein synthesis is required for memory (Flexner et al., 1964; Rossato et al., 2007). Accordingly, APP/PS1 mice present reduced brain protein synthesis in parallel to memory loss, and AβOs impair LTP-induced hippocampal protein synthesis (Ma et al., 2013). Nevertheless, the identity of memory-relevant translational products that are impacted in AD still remains to be determined.

Stress Kinase Activation

Cellular stress is also known to activate a family of protein kinases that mediate adaptive responses (Calay and Hotamisligil, 2013). These proteins are termed stress-sensitive kinases (or simply stress kinases) and include JNK, p38 MAPK, PKR, PERK and IKK, among other serine/threonine kinases (Vallerie and Hotamisligil, 2010; Hetz and Mollereau, 2014). Active stress kinases phosphorylate several protein targets to restore homeostasis. Nevertheless, their excessive or prolonged actions may trigger cell injury and, later, programmed cell death (Mattson, 2008; Vallerie and Hotamisligil, 2010; Hetz, 2012; De Felice et al., 2014).

Neuropathology studies have demonstrated abnormal activation of neuronal stress-sensitive kinases in AD brains. Indeed, abnormal phosphorylation of p38 MAPK (Hensley et al., 1999), JNK (Ma et al., 2009; Bomfim et al., 2012; Yoon et al., 2012), PERK (Hoozemans et al., 2005, 2009), PKR (Chang et al., 2002a; Paquet et al., 2011) and IKK (Talbot et al., 2012) have been reported in AD brains and might be core mediators of neuronal dysfunction. Accordingly, AβOs have been described to activate neuronal JNK and PKR to impair insulin signaling and synapse function (Ma et al., 2009; Bomfim et al., 2012; Lourenco et al., 2013), and transgenic animal models of AD exhibit similar alterations in JNK and PKR activity (Ma et al., 2009; Bomfim et al., 2012; Lourenco et al., 2013). Consistently,

blocking either PKR or the brain-enriched JNK3 rescue cognitive impairments in AD mouse models (Yoon et al., 2012; Lourenco et al., 2013), suggesting that stress kinase activation lies upstream of synapse and memory impairment in AD.

An attractive possibility is that PKR further drives the activation of other MAPKs, such as p38MAPK and JNK, thus exacerbating neuronal damage. Very recent findings suggest that the interaction between PKR and the RNA-binding protein TRBP is essential to promote eIF2α-P and JNK activation under obesity-induced metabolic stress (Nakamura et al., 2015). A similar scenario might also develop in AD even independently of TNF-α, given that oxidative stress has been reported to activate neuronal PKR (Mouton-Liger et al., 2012a). Moreover, AβOs could activate PKR in glial cells to instigate MAPK-dependent actions, exacerbating neuroinflammatory responses in AD brains. These notions still demand further investigation.

Neuroinflammation

Elevated markers of inflammation are found in both AD animal models and human AD brains (Ferreira et al., 2014; Monson et al., 2014; Heneka et al., 2015). Consistently, evidence for gliosis and central infiltration of peripheral immune cells is often found in histopathological studies in AD mouse models (Yamanaka et al., 2012; Lourenco and Ledo, 2013; Yang et al., 2013; Baik et al., 2014; Ferreira et al., 2014; Monson et al., 2014).

Amyloid aggregates (ranging from oligomers to fibrils) induce a neuroinflammatory profile that may lead to synapse and neuronal damage (Combs, 2009; Pan et al., 2011; Lourenco et al., 2013; Medinas and Hetz, 2013; Parajuli et al., 2013; Heneka et al., 2015). Nevertheless, rather than deposited plaques, AβOs are thought to be core inducers of brain inflammation, given that they are potent microglial activators (Floden and Combs, 2006; Dhawan et al., 2012; Ledo et al., 2013) and diffuse throughout brain regions (Lambert et al., 1998; Forny-Germano et al., 2014; Viola and Klein, 2015). Accumulating evidence suggests that AβO-induced microglial activation releases TNF-α and other cytokines that, in turn, act on neurons to cause stress signaling and synapse injury (Floden and Combs, 2006; Sondag et al., 2009; Bomfim et al., 2012; Dhawan et al., 2012; Lourenco et al., 2013).

Therefore, neuroinflammation is considered to take place over the degenerative course of AD and to be linked to cognitive dysfunction. In fact, our recent results showed that AβO-triggered elevations in TNF-α levels orchestrate neuronal stress mechanisms to impair brain insulin signaling (Bomfim et al., 2012), synapses and cognition in animal models of AD (Lourenco et al., 2013; **Figure 2**). This cascade is mediated by stress kinases, including JNK and PKR, in the brains affected by AβOs (Lourenco et al., 2013). Since evidence suggests that reducing neuroinflammation can counteract memory deficits in AD mouse models (Medeiros et al., 2007; McAlpine et al., 2009; Kiyota et al., 2010; Bachstetter et al., 2012), a more complete understanding of how brain inflammation develops may lead to effective targeting of aberrant mechanisms underlying cognitive symptoms in AD.

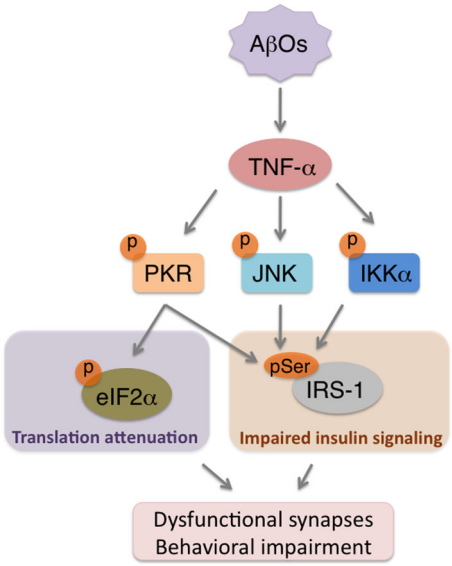

FIGURE 2 | AβOs trigger brain metabolic stress in Alzheimer's Disease (AD). Accumulation of AβOs in pre-AD brains instigates an inflammatory response that involves increased TNF-α production. TNF-α, in turn, acts on neurons to promote the activity of stress kinases (e.g., PKR, JNK, IKKα), which will serine phosphorylate both eIF2α-P to attenuate translation, and IRS-1 to impair insulin signaling. The combination of repressed protein synthesis and defective insulin signaling are components of a novel form of neuronal metabolic stress that may contribute to synapse deregulation and cognitive impairment in AD.

Metabolic Stress and Cognitive Function in AD

Experimental evidence has gathered inflammation, defective insulin signaling and cell stress to AD-linked neurotoxicity and neurodegeneration in a revised concept of metabolic stress (Paquet et al., 2011; Mouton-Liger et al., 2012a; Yoon et al., 2012; Ledo et al., 2013; Lourenco et al., 2013; Ma et al., 2013; Baleriola et al., 2014; De Felice et al., 2014). Although the classical alterations in glucose metabolism germane to metabolic impairments are observed in AD brains (Hoyer et al., 1988; Kapogiannis and Mattson, 2011; Chen and Zhong, 2013), the modern notion of metabolic stress also includes disturbances in proteostasis and activation of signaling pathways that mediate cellular stress.

In this context, the progressive build-up of AβOs in AD brains might trigger the activation of immune mechanisms, including glial cell reactivity and cytokine release that, in turn, lead to neuronal metabolic stress. A point of convergence of multiple stress pathways is found on elevated eIF2α-P levels. Accordingly, PKR, ER stress, eIF2α-P and ATF4 have been described as negative modulators of memory (Costa-Mattioli et al., 2007; Zhu et al., 2011; Rajasethupathy et al., 2012; Lourenco et al., 2013; Stern et al., 2013; Di Prisco et al., 2014; Ounallah-Saad et al., 2014). By acting together, such pathways might disrupt brain homeostasis and contribute to the cognitive decline observed in AD.

The precise mechanisms linking metabolic stress to synapse defects are still not fully understood, but the findings that increased eIF2α-P levels lead to LTP impairments (Ma et al., 2013) and synapse loss (Lourenco et al., 2013) in mice have provided initial clues on this causal relationship. Consistently, restoring normal brain eIF2α-P levels was shown to abrogate deficient levels of synaptic proteins and cognition (Lourenco et al., 2013; Ma et al., 2013), indicating a tight connection between eIF2α-P and synapse/memory integrity.

It is noteworthy that activation of PKR/eIF2α-P signaling (O'Connor et al., 2008; Devi and Ohno, 2010; Mouton-Liger et al., 2012a), as well as high-fat diet-induced metabolic stress (Wang et al., 2013) was shown to promote amyloidogenesis in a feed-forward cycle that might exacerbate amyloid pathology. It is thus tempting to speculate that accumulating injuries throughout life, including infections, diabetes and obesity, could instigate a brain metabolic stress scenario that includes ER stress and neuroinflammation to facilitate Aβ accumulation and sporadic AD onset at later stages of life (Herrup, 2010; Mattson, 2012; De Felice, 2013).

An unresolved question relates to whether brain insulin resistance could itself trigger AD-related phenomena, even in the absence of inflammation. In this regard, early studies using neuronal insulin receptor knockout (NIRKO) mice found that deficient brain insulin signaling causes abnormal tau phosphorylation without spatial memory impairment (Schubert et al., 2004). Recently, NIRKO mice were shown to develop anxiety and depressive-like behavior linked to altered dopamine metabolism (Kleinridders et al., 2015), and deletion of a single gene copy that encodes an insulin receptor subunit in the brain impairs synaptic plasticity and cognition (Nisticò et al., 2012). Nonetheless, it remains to be determined whether such mice develop brain metabolic stress in the presence or absence of neurotoxic stimuli. Future investigation may dissect the molecular steps that are required for metabolic stress-induced synapse impairments in an AD context.

Conclusions

Recent exciting evidence has connected AβO-induced neuronal stress to cognitive impairments in AD, in a mechanism that includes cytokine-induced activation of stress kinases and ultimately leads to neuronal and synapse dysfunction in AD experimental models (De Felice et al., 2007; Yoon et al., 2012; De Felice, 2013; Lourenco et al., 2013; Ma et al., 2013; Baleriola et al., 2014; De Felice and Ferreira, 2014; Ferreira et al., 2014). Hence, the combination of inflammation, neuronal insulin resistance, oxidative/ER stress and translational repression might generate a noxious scenario of brain metabolic stress to mediate and propagate synapse defects, resulting in cognitive deficits. In this context, ER stress and abnormal eIF2α-P levels emerged as key players in neuronal damage.

Sporadic AD is largely idiopathic, and it is noteworthy that Aβ-centric views of AD pathogenesis remain controversial (see Morris et al., 2014 for a critical review). Nonetheless, recent progress summarized here may have deep implications for disease prevention, as avoiding harmful events throughout life might reduce the risk of brain inflammation, metabolic stress and, consequently, of developing AD at later stages of life. Interrupting deleterious molecular pathways at prodromal stages will likely be the ideal strategy to delay AD progression. The identification of common AD drivers is imperative to establish effective therapeutics, and blocking neuronal metabolic stress at the earliest cognitive symptoms could offer a promising approach to minimize neuronal dysfunction and AD progression. Repurposing labeled anti-diabetic compounds could constitute an interesting option as they have been shown to attenuate AD-linked brain metabolic stress and memory dysfunction (Craft, 2012; De Felice et al., 2014). Future clinical trials may reveal whether these drugs, alone or in combination, are indeed effective in AD.

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REVIEW
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Menopause, obesity and inflammation: interactive risk factors for Alzheimer's disease

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Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder, the development of which is regulated by several environmental and genetic risk factors. Two factors theorized to contribute to the initiation and/or progression of AD pathogenesis are age-related increases in inflammation and obesity. These factors may be particularly problematic in women. The onset of menopause in mid-life elevates the vulnerability of women to AD, an increased risk that is likely associated with the depletion of estrogens. Menopause is also linked with an abundance of additional changes, including increased central adiposity and inflammation. Here, we review the current literature to explore the interactions between obesity, inflammation, menopause and AD.

Keywords: adiposity, aging, Alzheimer's disease, estrogen, hormone therapy, inflammation, obesity

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Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease that is the leading cause of dementia. The causal factor(s) that drives development and progression of the disease is still debated, though the primary agents are likely β-amyloid protein (Aβ) and the microtubule associated protein tau (LaFerla, 2010; Morris et al., 2014). Aβ is a small, soluble peptide normally found at low levels in brain and bodily fluids. Increased production and/or decreased clearance of Aβ fosters its inherent ability to self-associate into neurotoxic oligomeric forms that become deposited in the brain parenchyma as plaques and in the cerebrovasculature as cerebral amyloid angiopathy (Mucke and Selkoe, 2012). Interestingly, genetic mutations that underlie familial AD yield increased Aβ production or its propensity to aggregate (Tanzi, 2012). Thus, given both the toxic nature of Aβ and its genetic links to the disease, Aβ accumulation is widely theorized to be the key regulator of AD pathogenesis (Hardy and Higgins, 1992; Hardy, 2006). However, increasing evidence indicates an essential role of tau, which undergoes hyperphosphorylation resulting in the formation of neurofibrillary tangles, a hallmark of AD neuropathology found in many dead and dying neurons (Iqbal et al., 2010). Emerging evidence indicates that tau, like Aβ, can be a potent pathogenic protein and that it is capable of spreading pathology in a prion-like manner (Bloom, 2014; Zempel and Mandelkow, 2014).

AD is more than just the accumulation of oligomeric and fibrillar Aβ and abnormally phosphorylated tau. The disease is characterized by many pathologic changes, including hypometabolism (Mosconi et al., 2008; Yao et al., 2011), blood-brain-barrier (BBB) disruption (Zlokovic, 2011), and glial activation (Mrak and Griffin, 2005; Prokop et al., 2013). Sporadic AD, which is not driven by the genetic mutations in familial AD and represents the vast majority of cases, is likely to reflect the interactive effects of normal aging with numerous environmental risk factors and subtle genetic polymorphisms. In turn, these

interactions cooperatively disrupt pathways that regulate Aβ, tau, and other AD pathologies. Thus, unraveling AD risk, and perhaps developing successful prevention and intervention strategies, will require understanding the interactions between aging and a set of risk factors.

Two important AD risk factors are obesity and inflammation. Obesity has increased at an alarming rate across westernized countries, with approximately 70% of the US adult population currently classified as either overweight or obese (Ogden et al., 2014). Elevated adiposity increases the risks of numerous conditions, including metabolic syndrome (McGill, 2014), hypertension (Rahmouni, 2014), cardiovascular disease (Rocha and Libby, 2009), and AD (Jayaraman and Pike, 2014). One consequence of obesity that may underlie its pathogenic roles is chronic inflammation, which is observed both in brain and systemically. Inflammation is independently associated with AD and numerous other age-related disorders (Michaud et al., 2013; Bettcher and Kramer, 2014). A wealth of genetic, epidemiological, and experimental findings identifies pro-inflammatory pathways as significant regulators of various AD pathologies (Wyss-Coray and Rogers, 2012). Interestingly, risk factors can affect men and women differently. For example, the apolipoprotein E ε4 allele (ApoE4), the most significant genetic risk factor for late-onset AD, affects women much more strongly than men (Altmann et al., 2014). The enhanced vulnerability of aging women to some conditions, including increased risk of osteoporosis, cardiovascular disease and perhaps AD, is related to depletion of estrogens at menopause. Here, we consider the roles of obesity and inflammation in AD pathogenesis with a specific emphasis on women, who are disproportionately affected by AD.

AD and Obesity

Obesity is a precursor condition for numerous disorders, including cardiovascular disease, metabolic syndrome, and type 2 diabetes (T2D; Bonomini et al., 2015; Kim and Feldman, 2015). More recently, obesity and its associated comorbid conditions have been identified as significant risk factors for both cognitive decline and the development of AD (Jayaraman and Pike, 2014). A growing literature suggests that the insulin resistance and dysregulation of insulin signaling associated with T2D are precursors to both cognitive impairment and AD (Yaffe et al., 2004; Profenno et al., 2010). T2D in humans has been shown to increase the rate of age-related mental decline (Hassing et al., 2004), and those with T2D have been shown to develop cognitive impairment earlier than those without this risk factor (Cigolle et al., 2011). Further, adults with T2D have a significantly increased risk of AD (Luchsinger et al., 2004; Biessels et al., 2006; Profenno et al., 2010). Similarly, obesity appears to significantly increase AD risk, particularly when it is present during middle age (Kivipelto et al., 2005; Beydoun et al., 2008; Fitzpatrick et al., 2009; Xu et al., 2011). In contrast to most of the literature, a recent study with a very large sample size reported that midlife obesity (measured as body mass index, BMI) reduces dementia risk (Qizilbash et al., 2015). The absence of concordance among several well-controlled studies suggests that the relationship between obesity and AD is more complex than simply an increase in adiposity. For example, some studies suggest that dementia risk is not adversely affected by BMI *per se*, but rather by central obesity specifically (Whitmer et al., 2008; Gustafson et al., 2009; Luchsinger et al., 2012), which is often closely associated with adverse health effects including cardiovascular disease. In fact, cardiovascular outcomes are predicted better by measures of central obesity and often poorly by BMI (Yusuf et al., 2005; Dallongeville et al., 2012). Age is also an important consideration, as obesity in late life appears to reduce rather than increase AD risk (Fitzpatrick et al., 2009; Gustafson et al., 2009). Collectively, these observations suggest that central obesity in midlife induces changes that may create a neural environment conducive to initial development of AD pathology.

In general agreement with the human literature, experimental studies in mouse models of AD demonstrate that diet-induced obesity (DIO) significantly exacerbates AD-like neuropathology and worsens cognitive impairment (Julien et al., 2010; Herculano et al., 2013). Short-term high fat diet in an AD mouse model was shown to cause mild metabolic dysfunction and significant cognitive impairment, although no changes were observed in levels of Aβ, a key protein in AD pathogenesis (Herculano et al., 2013). High fat and high sucrose diets have also been shown to affect tau accumulation, processing and hyperphosphorylation (Julien et al., 2010; Orr et al., 2014; Takalo et al., 2014). In 3 × Tg-AD mice, longer-term administration of a high fat diet (16 weeks) impaired cognitive performance in both male and female mice (Barron et al., 2013). In line with the behavioral deficits, both male and female 3 × Tg-AD mice showed increased Aβ burden in the hippocampus. Male and female non-transgenic rodents both exhibit decreased cognitive performance after exposure to high fat diet (Molteni et al., 2002; Farr et al., 2008; Granholm et al., 2008). In some cases, male mice are more vulnerable to diet-induced cognitive impairment (Hwang et al., 2010). Since males typically exhibit significantly more robust metabolic deficits in response to high-fat diets (Barron et al., 2013), this may suggest that some aspects of obesity-induced cognitive impairment are related to metabolic disturbances, including insulin resistance. However, since even short-term exposure (9 days) to diets high in fat (Murray et al., 2009) results in impaired spatial memory in rats, more immediate effects of diet must also contribute to cognitive deficits. Diets high in sugars can also yield negative outcomes in AD mouse models (Cao et al., 2007; Orr et al., 2014) and wild-type rats (Hsu et al., 2015). Together, these studies show that exposure to obesity-inducing diets results in both rapid and long-term neural changes that impair cognitive performance and accelerate development of AD-like pathology.

The mechanism(s) by which obesity increases AD risk and cognitive deficits is unknown, although numerous possibilities have been proposed (Jayaraman et al., 2014). One widely discussed concept is that AD risk is linked with changes in glucose metabolism and insulin signaling (Blázquez et al., 2014; de la Monte and Tong, 2014). Consistent with this position, a reduction in brain glucose metabolism has been shown to be a preclinical symptom of AD (Mosconi, 2005). This reduction appears to be associated with altered insulin

signaling. In obese patients, insulin resistance results in an elevated release of peripheral insulin, but insulin concentrations in the brain are reduced, likely due to a decrease in insulin transport across the BBB (Craft, 2007). Although insulin levels are decreased centrally, the situation is further exacerbated by altered insulin signaling in the brain that is less effective than under normal conditions (Anthony et al., 2006). Interestingly, intranasal administration of insulin has been shown to improve hippocampal dependent memory in patients with early stages of AD (Reger et al., 2008; Claxton et al., 2015). Perhaps in contrast to these observations, DIO in female 3 × Tg-AD mice accelerated Aβ accumulation and behavioral impairment in the absence of apparent changes in peripheral insulin levels and sensitivity (Barron et al., 2013). Collectively, these data suggest that obesity-induced insulin resistance and/or impaired insulin signaling can contribute to AD neuropathology although additional factors also play significant roles.

Other than insulin-related deficits induced by obesity, increased adiposity has other systemic effects that may contribute to induction and progression of AD. For example, increased adiposity elevates neuroinflammation (Thaler and Schwartz, 2010; Thaler et al., 2012; Jayaraman et al., 2014; Aguilar-Valles et al., 2015), which has been implicated as a pathologic mechanism in AD (Johnston et al., 2011; Verri et al., 2012; Wyss-Coray and Rogers, 2012). Systemic and central inflammation will be discussed in greater detail below. However, cerebrovascular inflammation in particular has been widely associated with both obesity (Tucsek et al., 2014) and AD (Yu et al., 2012; Takeda et al., 2013). In fact, vascular inflammation may precede AD, as a transgenic rodent model of obesity and AD showed cerebrovascular inflammation and cognitive deficits prior to the deposition of Aβ (Takeda et al., 2010). In this model, cerebral amyloid angiopathy was much greater in obese animals compared to lean ones. Other vascular risk factors that are associated with obesity, including hyperlipidemia and hypertension, have also been identified as AD risk factors (Kivipelto et al., 2005). Thus, the mechanisms by which obesity increases AD risk are presumably multifactorial and likely modulated by a constellation of interactive risk factors and comorbidities.

AD and Inflammation

Although inflammation has been implicated in AD pathogenesis for many years, the importance of its role has been increasingly appreciated in the past few years. As has been discussed in other recent reviews (Mandrekar-Colucci and Landreth, 2010; Wyss-Coray and Rogers, 2012), multiple lines of evidence link inflammation as a key contributor to both the initiation and progression of AD and identify it as a compelling therapeutic target. Much of the recent focus on inflammation comes from the linkage to AD of polymorphisms in genes that are expressed by microglia and or play a role in innate immune responses (Tanzi, 2012). Included in this list are CD33 (Bertram et al., 2008; Hollingworth et al., 2011; Naj et al., 2011) and triggering receptor expressed on myeloid cells 2 (TREM2; Leavy, 2015), which are expressed in microglia and contribute to phagocytosis and Aβ clearance. Other immune-related factors linked to AD are CR1 and clusterin (Harold et al., 2009; Lambert et al., 2009), components of the complement system.

Microglia are the key mediators of neuroinflammation, functioning as the brain's resident macrophages. In their role as brain immune sentinels, microglia constantly survey the neural environment for both normal and pathological disruptions. In their normal state, microglia are clearly beneficial, utilizing phagocytosis to remove unwanted or unneeded materials and to regulate homeostatic processes such as synaptic remodeling (Chen and Trapp, 2015). Further, they may have important neurotrophic roles as suggested by their secretion of brain-derived neurotrophic factor and insulin-like growth factor 1 (Parkhurst et al., 2013; Suh et al., 2013). When activated under more pathological conditions, microglia exhibit a wide range of responses that continue to be defined (Colton, 2009). Among these responses, microglia alter their phagocytic activities and can secrete large amounts of pro-inflammatory cytokines. Cytokine secretion can be aggravated when microglia are primed by an initial activating insult (Teeling and Perry, 2009). This type of priming response may contribute to interactions among comorbid conditions such as obesity. In this case, a high fat diet that results in obesity may represent the first hit on the microglia and Aβ accumulation the second, yielding an exaggerated response that may drive pathology.

The AD brain is characterized by extensive gliosis, including activation and increased numbers of both astrocytes and microglia as well as elevated levels of inflammatory cytokines. Aβ plaques, once formed, are rapidly (within 1–2 days) surrounded by microglia (Meyer-Luehmann et al., 2008). Aβ has been shown to promote the release of inflammatory cytokines from microglia (Wyss-Coray and Rogers, 2012). Interestingly, increased pro-inflammatory factors can result in greater amyloid precursor protein production, leading to even more Aβ release and creating a vicious cycle that further promotes AD pathology (Karran et al., 2011). This progression of pathology appears to yield multiple states of microglial activation and deactivation that presumably vary in their effects on discrete AD pathologies (Colton, 2009). For example, initial exposures of microglia to Aβ, while the microglia are in their resting anti-inflammatory state, can result in effective Aβ clearance. However, this insult induces an activation state associated with the release of pro-inflammatory cytokines like tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β). After microglia achieve a pro-inflammatory state, they may be less able to effectively phagocytose extracellular Aβ (Koenigsknecht-Talboo and Landreth, 2005). Similarly, microglia in an AD mouse model mice were shown to be anti-inflammatory early in the disease, but to be activated later after the pathology had progressed (Jimenez et al., 2008).

Obesity and Inflammation

It has been more than two decades since the link between inflammation and insulin resistance was first shown with an increase in TNFα levels in obese rodents (Hotamisligil et al.,

1993). Confirming an active rather than passive role in pathology, neutralization of TNFα in obese rats resulted in a restored response to insulin. This and other early studies spawned intense study on the role of numerous inflammatory factors in obesity, metabolic syndrome, and T2D. It is now clear that not only is chronic low grade inflammation a symptom of metabolic disorders, but also that both peripheral (Hotamisligil, 2006) and central (Thaler and Schwartz, 2010) inflammation contribute to the development and progression of obesity and its comorbidities.

Visceral fat, also known as central or abdominal fat, is thought to have the greatest correlation with metabolic dysfunction (Kissebah et al., 1982; Nieves et al., 2003). An increase in body weight results in a corresponding increase in the number of mature macrophages found in adipose tissue. Macrophages invade and surround necrotic adipocytes and secrete excess pro- and anti-inflammatory cytokines, including TNFα and interleukin 6 (IL-6). The release of proinflammatory cytokines by macrophages is thought to contribute to the glucose disruptions and insulin resistance that is seen in obese individuals. Indeed, if macrophages are specifically ablated, insulin and glucose homeostasis can be rapidly restored and levels of inflammatory cytokines in adipose tissue, muscle and serum reduced (Patsouris et al., 2008). Further, adipocytes themselves secrete leptin and IL-6 after the macrophage infiltration and further increase the inflammatory response due in obesity. Adipose tissue is a highly active immunological organ that contributes to the increased inflammatory response to weight gain.

The role of inflammation in obesity also includes key contributions from the brain. Obesity-induced increases in peripheral and circulating cytokine levels likely affect the brain in multiple ways. However, available evidence suggests that central inflammation begins prior to the onset of obesity and appears to contribute to the process (Thaler et al., 2013). Much of the data on central inflammation and obesity has been generated from DIO paradigms, in which increased adiposity results from maintaining rodents on high-fat diets. DIO recapitulates much of the human obesity condition and can be modified to include specialized fats and/or sugars. Rodent DIO models have shown that both central and peripheral inflammation are induced with high fat diet (De Souza et al., 2005; Milanski et al., 2009; Thaler et al., 2012). Inflammation in the hypothalamus in response to high fat diet occurs within hours and persists for a few days before subsiding only to return after about 2 weeks (Thaler et al., 2012). Peripheral inflammation takes longer to develop and usually coincides with an increase in adipose tissue mass. Interestingly, mice with IL-1β, IL-6, or TNFα receptor knocked out show a worsening rather than a reduction in both obesity and metabolic syndrome, suggesting that inflammation is not the only contributing factor to these afflictions.

Inflammatory cytokines, which are elevated by obesity in both adipose tissue and brain, appear to be involved in both promoting inflammation and mediating many of its deleterious effects. In the hypothalamus, DIO induces inflammation within 3 days of the initiation of high fat diet in rodent (Thaler et al., 2012). Short-term high fat diet results in an upregulation of activated microglia and astrocytes. In humans, increased gliosis that is similar to that characteristic of injury is seen in the medial basal hypothalamus of obese patients (Thaler et al., 2012). Consistent with their role in adverse neural outcomes, cortical glia cultured from obese mice retain their inflammatory phenotype and impair neuron survival and growth *in vitro* (Jayaraman et al., 2014). Numerous inflammatory factors have been shown to be upregulated by obesity, including IL-1β, IL-6, TNFα and interleukin 18 (IL-18; Vandanmagsar et al., 2011), but which ones are central the relationship between obesity and AD are unclear. Here, we consider a few of the factors that are affected by obesity with an emphasis on those that also have been implicated in AD.

Leptin

Leptin is an adipokine that modulates appetite and informs the brain about the availability of stored energy. Although leptin is thought of as a peptide hormone, it bears remarkable similarity to inflammatory cytokines, especially interleukin 2 (IL-2). Further, the leptin receptor is homologous to the type 1 cytokine receptors. Leptin secretion increases with increasing adiposity (Considine et al., 1996). In the hypothalamus, leptin regulates appetite. High levels of leptin normally induce satiety. However, obese individuals develop leptin resistance, attenuating its ability to properly regulate appetite. The increase in adipose tissue in obese individuals initially results in elevated leptin release, but the response to leptin is blunted and the satiety signal is not recognized. The mechanisms that underlie leptin resistance have not been fully elucidated, but it may be the result of disrupted leptin signaling pathways, decreased leptin transport across the BBB, or increased inflammation in the hypothalamus (Park and Ahima, 2015).

In addition to regulating appetite, leptin is a regulator of inflammation and the immune system. Leptin regulates both innate and adaptive immune responses and generally increases levels of pro-inflammatory cytokines (Conde et al., 2014). Initial evidence of this link emerged from the observation that mice with deficiencies in leptin signaling (*ob*/*ob* or *db*/*db*) exhibit impaired immune responses (Lord et al., 1998). On the other hand, leptin exposure increases the proliferation and activation of T lymphocytes and promotes a T helper 1 phenotype (Martín-Romero et al., 2000). Similarly, leptin activates components of the innate immune response. For example, leptin increases activation of monocytes, including their production of cytokines such as TNFα and IL-6 (Santos-Alvarez et al., 1999). Also, leptin can induce the expression of nitric oxide and prostaglandin estrogen 17β-estradiol (E2) in cultured macrophages (Raso et al., 2002). With signaling capacity both peripherally and centrally and release modulated by fat mass, leptin is well positioned to function as a mediator of interactions between the brain and peripheral inflammatory responses (Carlton et al., 2012), particularly in the context of obesity (Aguilar-Valles et al., 2015).

Leptin resistance is predicted to foster AD pathogenesis as normal leptin signaling is associated with reduced AD risk. Persons with mild cognitive impairment or AD show lower plasma levels of leptin than cognitively normal controls (Johnston et al., 2014). Cerebrospinal fluid (CSF) leptin levels can remain stable with progression to AD, but a reduction in leptin signaling in hippocampus suggests AD is associated with

attenuation of leptin actions (Maioli et al., 2015). Conversely, in a prospective study, elevated leptin levels were associated with greater cerebral brain volume and reduced dementia risk (Lieb et al., 2009). In experimental AD models, leptin administration has been shown to have therapeutic effects on both Aβ deposition (Fewlass et al., 2004) and tau phosphorylation (Greco et al., 2008). Such protective pathways may become dysfunctional in the AD brain, where leptin levels are elevated but expression of its receptor is downregulated (Bonda et al., 2014). Further, the leptin receptor protein was found localized to neurons containing neurofibrillary tangles and within plaques, a situation expected to disrupt its signaling capability. Indeed, phosphorylation of the leptin receptor, which is required for its activation, was reduced when the leptin receptor was found in tangle-bearing neurons (Bonda et al., 2014). Collectively, available data indicate that a loss of beneficial leptin signaling can contribute to AD, suggesting that leptin resistance may be a mechanism by which obesity affects AD risk.

TNFα

TNFα appears to be produced exclusively by macrophages in adipose tissue with no contribution from adipocytes (Weisberg et al., 2003). Both TNFα protein and mRNA are robustly upregulated in obesity, an increase that has been shown to correlate with the development of insulin resistance (Hotamisligil et al., 1995). An increase in abdominal adipose tissue in particular has been shown to correlate with increased TNFα release (Tsigos et al., 1999). After weight loss, a decrease in TNFα protein in adipose tissue and a decrease in serum insulin is observed (Hotamisligil et al., 1995).

The neural effects of TNFα, which is produced in brain by neurons and glia, can be both desirable and detrimental. Transiently elevated levels of TNFα are beneficial. For example, TNFα can recruit astrocytes and microglia to sites of injury and activate a glial response (Flynn et al., 2003). However, TNFα also exerts negative effects on neural plasticity, such as reducing levels of long-term potentiation (Beattie et al., 2002; Ferguson et al., 2008). Increased TNFα is also thought to promote AD pathogenesis. For instance, *in vitro* and *in vivo* evidence indicate that TNFα increases Aβ levels by increasing expression of BACE1, an enzyme that drives Aβ production (Yamamoto et al., 2007). Further, TNFα has been shown to inhibit the transport of Aβ out of the brain and into peripheral circulation where it can be eliminated (López et al., 2008). TNFα-mediated increases in Aβ can lead to a vicious cycle. Aβ not only increases expression of TNFα (Meda et al., 1995; Akama and Van Eldik, 2000) but also is able to activate the TNFα receptor TNFRI, which is upregulated in AD brains compared to normal controls (Li et al., 2004; Cheng et al., 2010). Further, Aβ-induced generation of TNFα is implicated in the neurotoxic effects of Aβ (Xie et al., 2002). Conversely, reducing TNFα may be neuroprotective. For example, TNFRI knockout in AD transgenic mice diminishes AD-like neuropathology (He et al., 2007).

IL-6

Both adipocytes and macrophages can generate and secrete IL-6 from adipose tissue. Greater adiposity results in increased IL-6 secretion. Serum IL-6 concentrations have been positively correlated with obesity, insulin resistance (Bastard et al., 2000; Kern et al., 2001), T2D (Pradhan et al., 2001), and cardiovascular disease (Plutzky, 2001). Multiple studies in both mice and humans have linked IL-6 to T2D as the cytokine with the strongest association with metabolic dysfunction (Kern et al., 2001; Pradhan et al., 2001). Although IL-6 production is associated with visceral fat (Fontana et al., 2007), the relationship between IL-6 and obesity is not straightforward. For example, IL-6 knockout mice develop obesity, elevated leptin, and altered glucose homeostasis with aging (Wallenius et al., 2002), suggesting that long-term IL-6 depletion can contribute to metabolic dysfunction. IL-6 administered peripherally in knockout mice, reduces body weight and leptin levels. However, IL-6 treatment is also associated with deleterious effects in several paradigms. For example, in mouse hepatocytes and a human hepatocarcinoma model, IL-6 disrupts insulin signaling by decreasing insulin's ability to activate Akt, a critical part of insulin's modulation of downstream metabolic effects (Senn et al., 2002).

As with several other pro-inflammatory cytokines, chronic elevation of IL-6 results in negative neural consequences. Cross-sectional and longitudinal data indicate that elevated plasma IL-6 in mid-life is associated with significant cognitive decline (Singh-Manoux et al., 2014). Several IL-6 genetic variants have been shown to significantly regulate AD risk (Papassotiropoulos et al., 1999; Chen et al., 2012; Flex et al., 2014). IL-6 has consistently been shown to be elevated in the brains, especially near Aβ plaques, and in the CSF of AD patients (Bauer et al., 1991; Blum-Degen et al., 1995). The astrogliosis and microgliosis triggered by AD results in increased IL-6 release from both of these cell types (Erta et al., 2012). IL-6 has been implicated in AD pathogenesis through several different mechanisms. For example, cultured cortical neurons show more damage when treated with both Aβ and IL-6. Further, in cultured hippocampal neurons, IL-6 has been shown to increase tau phosphorylation (Quintanilla et al., 2004).

AD, Menopause and Hormone Therapy

Since women are disproportionately affected by AD, there has been considerable interest in understanding differences in estrogen across the lifespan associated with AD risk. One approach to investigating this idea is to consider the effects of pregnancies, which yield a net decrease in lifetime estrogen exposure (Bernstein et al., 1985; Hankinson et al., 1995). Several studies have shown that women who have birthed children are more likely to suffer from cognitive impairment and AD than nulliparous women (Ptok et al., 2002; McLay et al., 2003; Colucci et al., 2006; Beeri et al., 2009). Men with or without children show no change in AD risk, so the difference cannot be due to environmental factors associated with child rearing (Ptok et al., 2002). Nulliparous women are therefore seemingly protected from cognitive decline by their greater lifetime exposure to estrogens.

Another approach to address this issue is to consider the potential effects of estrogen loss on AD risk. Most studies have found that surgical menopause *prior* to the development

of natural menopause significantly increases risks for cognitive decline and AD (Rocca et al., 2007, 2011; Phung et al., 2010; Bove et al., 2014). Conversely, oophorectomy *after* the age of natural menopause does not appear to significantly alter AD risk (Rocca et al., 2011; Imtiaz et al., 2014). The implication of these studies is that early loss of sex steroid hormones can accelerate the development of AD. However, although brain levels of estrogens are lower in women with AD than age-matched women without neurologic disease, this relationship has been reported for only for women older than age 80 years (Yue et al., 2005; Rosario et al., 2011). Thus, while low estrogen appears to be associated with AD, the timing of this relationship with respect to disease onset remains unsettled.

If the depletion of ovarian sex steroid hormones at menopause is a risk factor for AD, then maintenance of hormones would be predicted to reduce AD risk. Consistent with this idea, AD risk has been reported to be lowest in postmenopausal women with the highest endogenous E2 levels and greatest in those with low E2 levels (Manly et al., 2000). Another approach is to consider how AD risk is affected by treatment with estrogen-based HT. Initial observational studies generally found significantly reduced risk of AD in women with a history of hormone therapy (HT) use (Henderson et al., 1994, 1996), findings that were supported by subsequent prospective studies (Tang et al., 1996; Kawas et al., 1997; Zandi et al., 2002). Human studies have been mirrored by experiments in transgenic mouse models of AD, in which ovariectomy (OVX) induced loss of sex steroid hormones increases and treatment with E2 generally reduces AD-like neuropathology (Zheng et al., 2002; Yue et al., 2005; Carroll et al., 2007; Zhao et al., 2011).

Despite the apparent benefits of estrogen-based HT in reducing AD risk, discrepant clinical findings have demonstrated neural risks and questioned its benefits. Most importantly, results of the Women's Health Initiative (WHI), a large double-blinded, placebo-controlled clinical trial, indicated increased rather than reduced dementia in subjects randomized to HT treatment (Shumaker et al., 2003, 2004). There are numerous factors that may contribute to the discordance of estrogen's benefits across the many human and rodent paradigms, including formulation of the treatment, continuous vs. discontinuous delivery, and route of administration. Perhaps the most significant issue is the timing of treatment, with initiation of HT near the onset of menopause hypothesized to be critical for its neural efficacy (Maki, 2006). In the WHI, the mean age of subjects was about 65 years old, many years past the average onset of menopause at age 51. Studies in which HT was initiated at or near menopause have generally reported benefits rather than risks. For example, in a Danish study in which middle aged women were randomized to HT and placebo treatment groups, beneficial effects on cognitive function were observed more than 10 years after the cessation of the 2–3 year HT regimen (Bagger et al., 2005). This finding suggests that using HT, even for a short time during menopause, may have a positive effect on cognition much later. Similar results have been found for AD risk: HT is associated with decreased risk if delivered near menopause but has no benefit or even increases risk when started several years after menopause (Henderson et al., 2005; Whitmer et al., 2011; Shao et al., 2012). More work will be required to fully elucidate the relationship between HT and AD risk, but emerging results seem to suggest that short-term HT near menopause onset may offer a reasonable strategy to impede development of dementia.

Efficacy of HT is also likely to be modulated by genetic factors. The most significant genetic risk factor for late-onset AD is the ε4 allele of ApoE4. ApoE4 regulates lipid trafficking and may play a role in the removal of Aβ from the brain (Näslund et al., 1995; Leduc et al., 2011). HT may be differentially effective in women based on their ApoE status. In mice, E2 is able to reduce inflammatory markers in cultures from ApoE3, but not ApoE4 mice (Brown et al., 2008). One study showed that HT around menopause is more effective in preventing cognitive decline in ApoE4-negative women than those with even a single ApoE4 allele (Yaffe et al., 2000). Further, the ApoE4 genotype may increase the risk of AD more in females than males (Farrer et al., 1997; Bretsky et al., 1999). Increased AD risk in ApoE4 carriers requires only one ApoE4 allele in females with no further risk increase in homozygotes, whereas significant male risk appears to require two ApoE4 copies (Payami et al., 1996). A more recent study showed that both male and female ApoE4 carriers were at greater risk of AD with even one ApoE4 allele, but that the risk for female carriers was significantly greater (Altmann et al., 2014). How interactions between ApoE, HT and AD are modulated by obesity, inflammation, and other factors that drive AD pathogenesis remains to be determined.

Menopause and Adiposity

The shift from young adulthood into middle age is associated with increasing proportions of women that are overweight and/or obese (Ogden et al., 2014). This weight gain likely reflects multifaceted consequences of aging. There is evidence that depletion of sex steroid hormones during menopause can contribute to weight gain, although body weight also predicts subsequent changes in hormone levels (Guthrie et al., 1999; Sternfeld et al., 2004; Wildman et al., 2012). Significant increases in central fat have been reported with greater waist circumference after the last menstrual period (Poehlman et al., 1995; Björkelund et al., 1996; Ho et al., 2010). This increase in adiposity associated with menopause is linked with increased risks for obesity (Rachoń and Teede, 2010), metabolic syndrome (Carr, 2003; Cho et al., 2008) and T2D (Wajchenberg, 2000). Although increasing age may partially account for the increase T2D incidence in postmenopausal women (Janssen et al., 2008), the change in fat distribution has also been shown to be a contributing cause (Barrett-Connor et al., 1996; Tchernof et al., 1998; Sites et al., 2000).

Studies in both humans (menopause) and rodents (reproductive senescence) indicate that age-related ovarian hormone loss contributes to changes in the distribution of adipose tissue. The significant decrease in estrogen and progesterone that results from follicular depletion yields a more androgenic pattern of fat distribution—an increase in central or abdominal adiposity. OVX in mice, a model of surgical menopause, can result in a significant increase in body weight (Stubbins et al., 2012). This elevated weight results from a decrease in energy expenditure, as opposed to increases in

calorie consumption, and promotes insulin resistance, increased adipocyte size, and peripheral inflammation (Rogers et al., 2009). Treatment of mice with the E2 results in reductions in all of these outcomes.

In postmenopausal women, estrogen-based HT has been shown to decrease some of these metabolic effects in addition to restoring a more gynoid pattern of fat distribution (Barrett-Connor et al., 1996; Salpeter et al., 2006). HT can attenuate central adiposity when administered to early postmenopausal women (Haarbo et al., 1991; Ahtiainen et al., 2012). Additionally, HT may decrease total weight gain during menopause, especially in non-obese women (Kristensen et al., 1999). Further, HT has been shown to increase the effectiveness of concurrent lifestyle and pharmaceutical treatments for some obesity-related comorbidities (Golden et al., 2013).

The mechanisms by which E2 reduces obesity and risk of T2D remain to be fully elucidated. Paradoxically, risk of maternal insulin resistance is highest in late pregnancy when E2 levels are high (Ryan and Enns, 1988), yet low E2 levels after menopause have been linked with T2D (Carr, 2003; Meyer et al., 2011). Aromatase knockout mice, which cannot synthesize E2, are obese and insulin resistant (Jones et al., 2000). Estrogen receptor α (ERα) is likely to play a role in regulation of obesity as ERα knockout mice are more obese and glucose intolerant than wild-type females (Heine et al., 2000; Bryzgalova et al., 2006). Further, both peripheral and central E2 administrations have been shown to act through different pathways with similar overall outcomes of reductions in insulin resistance, glucose dysregulation and adiposity (Yonezawa et al., 2012). Peripheral and central E2 administration resulted in decreased adipocyte size and reduced expression of macrophage markers, although peripheral E2 treatment elicited greater changes. Only peripheral E2 administration decreased adipose expression of the pro-inflammatory cytokine TNFα. Central and peripheral E2 administration both increased energy expenditure, but central E2 caused much greater spontaneous locomotor activity. Thus, E2 likely regulates adiposity and metabolic outcomes by actions on several tissues. How these actions are affected by aging likely underlies the interactions between menopause, obesity and the efficacy of HT.

Inflammation and Menopause

There are extensive literatures on the relationships between estrogens and other sex steroid hormones and regulation of inflammation. In general, one can reasonably argue that estrogens function as potent anti-inflammatory factors. Thus, depletion of E2 at menopause results in elevated pro-inflammatory cytokines and may place tissues throughout the body at increased risk of inflammation and diseases associated with inflammation (Pfeilschifter et al., 2002). However, this is an extensive and complex literature as estrogens regulate several aspects of immune function and inflammation (Straub, 2007) and thus can protect against some conditions but promote others (Gilliver, 2010). The relationships between estrogens, menopause, and inflammation-related disorders have been well described in numerous reviews for several conditions, including cardiovascular disease (Camilleri et al., 2012; Knowlton and Lee, 2012), osteoarthritis (Martín-Millán and Castañeda, 2013), and rheumatoid arthritis (Islander et al., 2011). Thus, although estrogens can be broadly defined as anti-inflammatory, the effects of estrogen loss and estrogen-based HT depend upon the tissue, cell type, and context.

How menopause affects neuroinflammation has not been well studied. However, as with other tissues, the brain exhibits a generally pro-inflammatory phenotype with increasing age. In a study that examined changes in gene expression across age in adult human brain, male and female brains exhibited significant differences in the number of genes changing expression (higher in males), the patterns of changes in terms of gene function, and the brain region-specific nature of the changes (Berchtold et al., 2008). Of particular interest to the current topic, women exhibited greater proportional age-related increases in expression of genes associated with immune and inflammatory functions. Further, both women and men showed increased expression of these genes in hippocampus and entorhinal cortex (both of which are strongly affected in AD), but only women had significant increases in other brain regions (Berchtold et al., 2008), suggesting a more global pro-inflammatory condition in the aging female brain.

Like the human brain, the rodent female brain shows increased inflammation with aging that is regulated, in part, by estrogen status. For example, the frontal cortex of middle-aged female rats shows increased expression of several microglial and immune function genes as a consequence of both aging and estrogen-deprivation (Sárvári et al., 2012). Importantly, the authors also found a strongly overlapping pattern of gene expression changes in the frontal cortex of older postmenopausal women relative to younger premenopausal women. Confirming a protective role of estrogens, treatment of OVX middle-aged rats with E2 or ER-specific agonists significantly decreased expression of several microglial and immune function genes in the frontal cortex (Sárvári et al., 2011). This relationship extends to pro-inflammatory cytokines such as TNFα and IL-1β, which show increased hippocampal expression in female mice as consequences of both aging and OVX-induced estrogen depletion (Benedusi et al., 2012). The age-related increase in the pro-inflammatory state of the brain can be exacerbated by inflammatory challenges. For example, the increased hippocampal expression of cytokines induced by high cholesterol diet is significantly worsened in reproductively senescent female rats, an effect that can be attenuated by estradiol treatment (Lewis et al., 2010). However, the ability of E2 to protect against elevated cytokine levels can significantly diminish, and in some cases reverse, in aged female brain (Nordell et al., 2003). Collectively, these observations indicate that both aging and menopause contribute to increasing levels of neuroinflammation, which are predicted to cooperatively interact in the promotion of inflammation neural diseases such as AD.

Conclusion

AD neuropathology is characterized and likely driven by the accumulation of Aβ and abnormally phosphorylated tau. The development of these and other components of AD

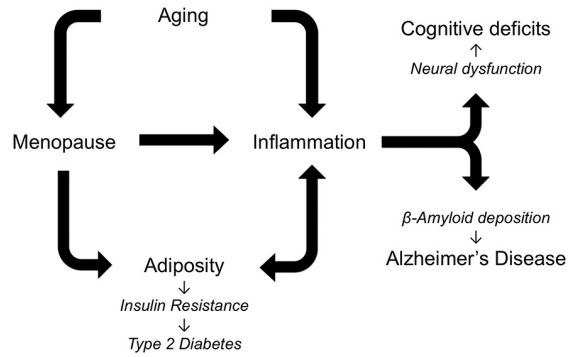

FIGURE 1 | Alzheimer's disease (AD) is a multifactorial disorder in which multiple risk factors are theorized to interact in regulating pathogenesis. As depicted in the diagram an essential factor in AD is increasing age, which is also associated with elevated inflammation and, in women, menopause. The loss of estrogens at menopause increases central adiposity, which in turn increases inflammation and predisposes women to metabolic syndrome, insulin resistance, and AD. Individually and cooperatively, aging, menopause, adiposity, and inflammation lead to cognitive deficits and AD.

neuropathology result from the interactive effects of several risk factors (**Figure 1**). Most importantly, AD is dependent upon aging. The negative outcomes of all genetic and environmental risk factors of AD require advancing age. In women, chronological aging is also tied to reproductive aging that is manifested as menopause in mid-life. Menopause results in cessation of the ovarian cycle and its cyclical production of estrogens and progesterone, sex steroid hormones with numerous protective roles against AD (Pike et al., 2009). Beyond protective actions against specific aspects of AD neuropathology, estrogens function throughout the body to guard against the development of increasing central adiposity and inflammation, both of which are implicated in the initiation and progression of AD. Of course, aging is also associated with significant increases in inflammation and central obesity, independent of estrogen levels. Thus, women are simultaneously exposed to the consequences of both chronological and reproductive aging, both of which can function as drivers of AD, obesity, and inflammation.

Estrogen-based HT initiated prior to the end of menopause has been shown to be effective in combating both the increased adiposity and the cognitive decline associated with aging. Although HT can exhibit adverse side effects, especially in those women who may be predisposed, emerging results suggest that relatively short-term HT initiated near the onset of menopause is likely to yield largely beneficial effects for most women. Much more research will need to be done to determine precisely which genetic or environmental factors define the target population.

Inflammation is another seemingly invariant consequence of aging. In females, inflammation is further increased by the loss of estrogens at menopause. As with many other age-related diseases, AD is linked with elevated inflammation during aging by abundant data from several fields. Several specific inflammatory factors have been linked to both obesity and AD including leptin, TNF-α and IL-6. Since estrogens protect against both the development and deleterious consequences of inflammation across many tissues, depletion of estrogens at menopause is theorized to contribute to women's vulnerability to AD. Age-related increases in adiposity and changes in the distribution of fat to central depots further increase the risk of obesity, which promotes downstream conditions including insulin resistance, metabolic syndrome and T2D. Thus, a highly interactive set of relationships develops in women: the cumulative effects of aging, menopause, adiposity, and inflammation result in increased risk for neural dysfunction and AD pathogenesis (**Figure 1**). Certainly this set of factors is incomplete, as new data continue to identify additional factors, including bioenergetics, that not only are altered during reproductive aging but also can promote AD (Yin et al., 2015). Continued research is needed to address many unresolved issues about these pathological interactions, including how they are modified by genetic risk factors such as Apolipoprotein E (ApoE) and the extent to which they can be effectively attenuated by estrogen-based HT.

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frontiers in Aging Neuroscience

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C-reactive protein, advanced glycation end products, and their receptor in type 2 diabetic, elderly patients with mild cognitive impairment

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Objective: The aim of the study was to evaluate serum levels of advanced glycation end products (AGEs), receptor for advanced glycation end products (RAGE), and C-reactive protein (CRP) in elderly patients with type 2 diabetes mellitus with and without mild cognitive impairment (MCI) and to determine the predictors (including AGEs, RAGE, and CRP levels) of having MCI in elderly patients with type 2 diabetes.

Methods: Two hundred seventy-six diabetics elders were screened for MCI (using the Montreal Cognitive Assessment: MoCA score). Data of biochemical parameters and biomarkers were collected.

Results: Serum AGEs, RAGE, and CRP levels were significantly increased in MCI patients compared to controls. In group of patients with MCI, serum RAGE level was positively correlated with AGEs level and with CRP level. RAGE, AGEs, and CRP concentrations were positively correlated with HbA1c levels and negatively correlated with MoCA score. The univariate logistic regression models revealed that variables, which increased the likelihood of diagnosis of MCI in elderly patients with type 2 diabetes were higher levels of HbA1c, RAGE, AGEs, CRP, TG, lower level of HDL cholesterol, previous CVD, HA, or use of HA drugs, hyperlipidemia, retinopathy, nephropathy, increased number of co-morbidities, older age, and less years of formal education. HA or use of HA drugs, previous CVD, higher level of RAGE and CRP, older age and less years of formal education are the factors increasing the likelihood of having MCI in elderly patients with type 2 diabetes in multivariable model.

Conclusion: In summary, serum AGEs, RAGE, and CRP are increased in the circulation of MCI elderly diabetic patients compared to controls. A larger population-based prospective study needs to be performed to further confirm the relationship between AGEs, RAGE, and the cognitive decline or progress to dementia.

Keywords: AGEs, cognitive impairment, diabetes, elderly, RAGE

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INTRODUCTION

Diabetes mellitus is one of the important chronic diseases worldwide.

The prevalence of type 2 diabetes mellitus (T2DM) raised rapidly in the last few decades (Wild et al., 2004). Complications include small and large vessels, eyes, kidneys, cranial, and peripheral nerves. Recent studies suggested that diabetes is a risk factor for cognitive decline and dementia in the elderly (Cukierman et al., 2005; Gorska-Ciebiada et al., 2009, 2014). T2DM has a highly complicated pathogenesis, so the etiology of cognitive impairment in diabetes is probably associated with many factors (Strachan et al., 1997). One of the recent hypotheses suggests that low-grade chronic inflammation is associated with mild cognitive impairment (MCI) or with cognitive decline (Yaffe et al., 2003; Schuitemaker et al., 2008). MCI is a transitional stage between normal cognitive aging and dementia. One large study has proven that some inflammatory parameters, such as C-reactive protein, TNF-α, and IL-6, are connected with cognitive dysfunction and decline (Yaffe et al., 2003). There are evidences from other population-based study that there are significant associations between CRP and MCI and with the attention and executive function domain score (Roberts et al., 2009). Another potential marker of cognitive dysfunction is a receptor for advanced glycation end products (RAGE). RAGE is a transmembrane receptor that belongs to the immunoglobulin super family of cell surface receptors. It interacts with its ligands and causes persistent inflammatory responses at vascular wall and vascular injuries, a major complication of diabetes (Neeper et al., 1992; Hori et al., 1996; Schmidt et al., 2000a; Kumano-Kuramochi et al., 2009). The major RAGE ligands in diabetes are advanced glycation end products (AGEs). They are derivatives of proteins, lipids, and ribonucleic acids. Serum AGEs levels increases with age and with diabetes or hyperglycemic states (Ulrich and Cerami, 2001). RAGE-mediated mechanisms play a crucial role in diabetes complications because they induce reactive oxygen species-mediated inflammation in vascular wall (Win et al., 2012). RAGE is also an important cell-signaling receptor involved in cognitive impairment. Recent studies report the association between RAGE levels and cognitive impairment in Alzheimer's disease (Deane and Zlokovic, 2007). Increased expression of RAGE was found in neurons and microglia in the hippocampus (Lue et al., 2001). Some authors have suggested that RAGE and other receptors could be involved in the injury of the brain in Alzheimer's disease and diabetes by microglial activation and oxygen-mediated neuroinflammation (Lue et al., 2012).

Data concerning serum AGEs and RAGE levels in MCI subjects with diabetes are lacking; therefore, the aim of the study was to (1) evaluate serum levels of AGEs, RAGE, and CRP in elderly patients with T2DM with and without MCI and (2) determine the predictors (including AGEs, RAGE, and CRP levels) of having MCI in elderly patients with T2DM.

MATERIALS AND METHODS

Study Population

A survey was conducted among unselected 276 elders who attended to outpatient clinic belonged to the Department of Internal Medicine and Diabetology, University Hospital no 1 in Lodz, Poland. A brief screening for recruitment was conducted by the investigators to identify potential participants. We included patients aged 65 and over with diabetes type 2 diagnosed minimum 1 year earlier, subjects who had been able to understand and cooperate with study procedures. The exclusion criteria were diagnosed depression or dementia, use of possible or known cognition-impairing drugs in the previous 3-month, presence of neoplasm, constant alcohol or substance abuse, severe visual, mobility, or motor coordination impairment, history of head trauma, inflammatory or infectious brain disease, severe neurological or psychiatric illness (**Table 1**).

Written informed consent was obtained from the participants at the beginning of the study. The first part of visit included a morning blood draw after a 10- to 12-h overnight fast, blood pressure measurements, height and weight assessment, and complete physical examination. Then, patients had eaten a breakfast followed by capillary glucose level measuring to ensure that participants were not hypoglycemic at the time of cognitive testing. The second part of visit took place in a private area in the clinic. Subjects completed a questionnaire describing baseline demographics and underwent cognitive testing.

Participant Characteristics, Clinical Evaluation, and Risk Factor Assessment

Demographic variables and possible risk factors were recorded in a standardized interview. Weight and height were measured to calculate body mass index [BMI = weight/height^2 (kg/m^2)]. The systolic and diastolic blood pressures (millimeter of mercury) were measured with the patient in sitting position after 5 min of rest. The detailed medical history of diabetes type 2 was taken and includes diabetes duration, currant treatment for diabetes and complications if present, family history of diabetes, co-morbid diseases of the patient [hyperlipidemia, hypertension, cardiovascular disease (CVD), lung disease, cancer, gastrointestinal tract diseases] and their treatment. Educational level was recorded in years of education. Smoking was classified as current, past, or never. Physical activity was recorded if any present. Diabetic vascular complications were assessed based on the existence of nephropathy, retinopathy, neuropathy, CVD, and stroke. Hypertension was defined as

TABLE 1 | The inclusion and exclusion criteria of the study.

The inclusion criteria
Age ≥65 years
Diabetes type 2 diagnosed minimum 1 year earlier
Full ability to understand and cooperate with study procedures
The exclusion criteria
Diagnosed depression or dementia
Use of possible or known cognition-impairing drugs in the previous 3-month
Presence of neoplasm
Constant alcohol or substance abuse
Severe visual, mobility, or motor coordination impairment
History of head trauma
Inflammatory or infectious brain disease
Severe neurological or psychiatric illness

either a history of hypertension or use of any antihypertensive agents, Hyperlipidemia defined as use of any lipid lowering agent or an untreated serum LDL cholesterol level 2.6 mmol/l or/and triglycerides 1.7 mmol/l.

Blood Biochemistry

After overnight fasting, blood samples were taken by venipuncture to assess serum levels of glycosylated hemoglobin (HbA1c), total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). All the parameters were measured in a centralized laboratory.

Determination of Serum AGEs, RAGE, and CRP

The serum levels of AGEs, RAGE were assessed using ELISA kit (EIAab, Wuhan, China), and CRP was determined by Quantikine Human Immunoassay ELISA kit (R&D System, Minneapolis, MN, USA) according to the instructions of the manufacturer. Minimum detectable concentrations were 0.78 ng/ml for AGEs and 4.12 pg/ml for RAGE, 1×10^{-5} mg/l for CRP.

Neuropsychological Evaluations

All participants underwent the following tests: the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005) to evaluate the cognitive impairment, long version of the geriatric depression scale (GDS-30) (Yesavage et al., 1982–1983) to assessed the depressive mood, Katz Basic Activities of Daily living (BADL) and Lawton instrumental activities of daily living (IADL) questionnaires to collect information on daily activities (Lawton and Brody, 1969; Katz et al., 1970). The MoCA tests 8 cognitive domains, visual–spatial ability, attention, executive function, immediate memory, delayed memory, language, abstraction, calculation, and orientation, for a maximum total score of 30. The normal MoCA score is ≥26, with one point added if the subject has fewer than 12 years of formal education. The MoCA is better than other tools to detect MCI in the elderly patients with type 2 diabetes (Alagiakrishnan et al., 2013). MCI was diagnosed based on criteria established by the 2006 European Alzheimer's Disease Consortium, which are currently available standard test (Petersen, 2004; Portet et al., 2006). These criteria included absence of dementia. The cut-off points for MoCA scores (19/30) are recommended for the diagnosis of "dementia" in epidemiological studies. Patients with score 19 and below were excluded from the study as dementia and sent to psychiatrist for further care. The criteria mentioned above included also absence of major repercussions on daily life (in our study, measured by Katz BADL and Lawton IADL).

This interview was followed by GDS for mood assessment (Yesavage et al., 1982–1983). GDS consists of 30 items. Scores ranging from 0 to 9 were considered as normal, and 10 to 19 were considered to have depressive symptoms. Score 20 and above was excluded from the study as severe depressive symptomatology and sent to psychiatrist for further diagnosis.

According to criteria mentioned above 276 older subjects with diabetes type 2 were selected into groups: patients with MCI and controls (patients without MCI).

Ethics

The study was operated in accordance with the World Medical Association's Declaration of Helsinki. Each participant was assigned a number by which he/she was identified to keep his or her privacy. The approval was obtained from the independent local ethics committee of Medical University of Lodz No RNN/420/13/KB. The purpose, nature, and potential risks of the experiments were fully explained to the subjects, and all subjects gave written informed consent at the beginning of the study. The subjects had the full capacity to consent because they maintained general cognitive function and daily activities. We included only patients who had been fully able to understand and cooperate with study procedures. We excluded subjects with diagnosed depression or dementia. There was not any surrogate consent procedure (e.g., whereby next of kin or legally authorized representative) consented on the behalf of participants.

Statistical Analysis

All data are presented as means ± SD. Normality of distributions was assessed using the Shapiro–Wilk tests. The descriptive statistics the continuous variables using the Student's *t*-test or the Mann–Whitney *U* test whenever applicable and non-continuous variables using χ^2 test. Pearson correlation analysis for normally distributed variables and Spearman rank correlation for non-normally distributed variables were used to assess relationships. Simple logistic regression model was done in order to select the so-called independent factors that increase the selection risk of MCI in elderly patients with type 2 diabetes. Then, multivariable regression model was done in order to select the "strongest" factor from independent risk factors. To "optimize" the multivariable model, a stepwise approach was used (backward elimination with Wald criteria). Odds ratios (OR) were computed and presented with the 95% interval of confidence (CI). A p-value of <0.05 was considered statistically significant. Statistica 10.0 (StatSoft, Poland, Krakow) was used for analysis.

RESULTS

General Description of MCI Subjects and Controls

Table 2 describes the baseline characteristics of the study group. Compared with controls patients with MCI were significantly older, less educated, had a longer duration of diabetes, more were diagnosed with CVD, hypertension, hyperlipidemia, retinopathy, nephropathy, and other co-morbidities. MoCA score was significantly lower in subjects with cognitive impairment. The mean level of HbA1c and triglycerides was significantly higher, and level of HDL was lower in patients with MCI compared to controls. Furthermore, CRP was found to be increased in MCI patients compared to control group ($p < 0.001$). Lastly, there were no significant differences between the groups in sex, BMI, history of smoking, stroke, presence of neuropathy, depressive syndrome, type of the treatment, systolic and diastolic blood pressure, the plasma levels of fasting glucose, and total and LDL cholesterol ($p > 0.05$).

TABLE 2 | Demographic and clinical characteristics of type 2 diabetic elderly patients.

	All subjects	MCI	Controls	χ^2/Z	*p*-value
Number of patients	276	87	189		
Age (years)*	73.6 ± 4.8	75.7 ± 4.6	72.6 ± 4.6	−4.96	<0.001
Gender (female/male)	149/127	53/34	96/93	2.46	0.12
Education-years*	11.3 ± 2.4	9.7 ± 1.8	12.0 ± 2.2	7.97	<0.001
Smoked tobacco regularly	93 (33.7%)	26 (29.8%)	67 (35.4%)	0.83	0.36
Duration of T2DM (years)*	8.69 ± 6.23	11.25 ± 6.3	7.51 ± 5.85	−5.96	<0.001
Microvascular complications Retinopathy (%)*	121 (43.8%)	61 (70.1%)	60 (31.7%)	35.6	<0.001
Nephropathy (%)*	97 (35.1%)	43 (49.4%)	54 (28.5%)	11.37	0.007
Neuropathy (%)	56 (20.2%)	20 (22.9%)	36 (19.04%)	0.57	0.45
Macrovascular complications Previous CVD (%)*	109 (39.5%)	71 (81.6%)	38 (20.1%)	94.3	<0.001
Stroke (%)	14 (5.07%)	7 (8.04%)	7 (3.7%)	2.33	0.13
Previous HA/use of HA drugs (%)*	213 (77.2%)	80 (91.95%)	138 (73.01%)	18.3	<0.001
Hyperlipidemia (%)*	218 (78.9%)	81 (93.1%)	132 (69.8%)	12.87	<0.001
Co-morbidity (*n*)*	4.66 ± 3.11	7.07 ± 3.22	3.55 ± 2.33	−8.15	<0.001
Depressive syndrome (%)	82 (29.7%)	25 (28.7%)	57 (30.2%)	0.06	0.81
Treatment Insulin (%)	130 (47.1%)	42 (48.2%)	88 (46.5%)	0.07	0.79
OAD (%)	222 (80.4%)	71 (81.6%)	151 (79.8%)	0.11	0.74
MoCA score*	25.6 ± 3.07	21.6 ± 1.5	27.4 ± 1.3	13.34	<0.001
BMI (kg/m^2)	29.9 ± 3.67	30.4 ± 3.59	29.6 ± 3.68	−1.92	0.054
Systolic blood pressure (mmHg)	136.2 ± 15.9	136.5 ± 16.4	136.05 ± 15.8	−0.25	0.79
Diastolic blood pressure (mmHg)	75 ± 7.9	75.1 ± 8.0	74.9 ± 7.8	−0.09	0.92
Fasting plasma glucose (mmol/l)	129.3 ± 26.1	129.8 ± 27.2	129.1 ± 25.6	−0.14	0.88
HbA1c (%)*	7.24 ± 0.68	7.73 ± 0.71	7.01 ± 0.54	−7.5	<0.001
Serum cholesterol (mmol/l)	10.3 ± 2.18	10.31 ± 2.2	10.29 ± 1.71	−0.5	0.61
Serum LDL-C (mmol/l)	6.06 ± 1.67	6.01 ± 1.64	6.08 ± 1.73	−0.2	0.86
Serum triglycerides (mmol/l)*	9.65 ± 2.23	10.59 ± 2.68	9.22 ± 1.84	−6.6	<0.001
Serum HDL-C (mmol/l)*	2.5 ± 0.51	2.3 ± 0.6	2.67 ± 0.42	6.34	<0.001
CRP (mg/L)*	5.08 ± 2.8	7.6 ± 2.7	3.9 ± 2.0	−9.79	<0.001
AGEs (ng/ml)*	1.4 ± 1.06	2.19 ± 1.12	1.04 ± 0.82	−8.1	<0.001
RAGE (ng/ml)*	2.67 ± 1.68	4.24 ± 1.89	1.94 ± 0.91	−9.7	<0.001

**Significance, $p < 0.05$; comparing patients with MCI and those without MCI (controls).*

T2DM, diabetes type 2; OAD, oral anti-diabetic drug; CVD, cardiovascular disease; HA, hypertension; BMI, body mass index; CHOL, total cholesterol; CRP, C-reactive protein; AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end products; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MoCA, Montreal Cognitive Assessment,

Data are mean – SD values. Mann–Whitney U test (Z), or χ^2 test was used to test for significant differences.

Serum RAGE, AGEs, and CRP in MCI and Controls

Serum RAGE and AGEs levels were significantly increased in MCI patients compared to controls ($p < 0.001$). As expected, in group of diabetic elderly patients with MCI serum RAGE level was positively correlated with AGEs level ($r = 0.85$, $p < 0.001$) and with CRP level ($r = 0.54$, $p < 0.001$) (**Figures 1A–C**). Furthermore, RAGE, AGEs, and CRP concentrations were highly correlated with HbA1c levels (**Figures 2A–C**). We found also positive but weak correlation between these parameters and triglycerides levels and negative correlation with HDL levels. The results indicated that MoCA score was negatively correlated with RAGE, AGEs, and CRP levels (**Figures 3A–C**). Data are presented in **Table 3**.

Logistic Regression Models

Because many factors can influence the results we constructed the univariate logistic regression models and finally multivariable regression model to determine the predictors of having MCI in elderly patients with type 2 diabetes. The independent variables entered in the model at step one were demographic variables (age, gender, education), duration of diabetes, glycemic control (HbA1c level), CVDs (MI, angina, stroke), cardiovascular risk factors (BMI, smoking status, hyperlipidemia, previous HA or use of HA drugs), microvascular complications, presence of depressive syndrome, number of co-morbid conditions, levels of total, LDL, HDL cholesterol, triglycerides, AGEs, RAGE, and CRP. The univariate logistic regression models revealed that variables which increased the likelihood of having been diagnosed with MCI in elderly patients with type 2 diabetes were higher levels of HbA1c, RAGE, AGEs, CRP, TG, lower level of HDL cholesterol, previous CVD, HA or use of HA drugs, hyperlipidemia, retinopathy, nephropathy, increased number of co-morbidities, older age, and less years of formal education (**Table 4**).

Table 5 shows the results of modeling the risk of having MCI by multivariable regression. All variables presented in **Table 3** were introduced to this model. The multivariable model was optimized by the stepwise approach. HA or use of HA drugs, previous CVD, higher level of RAGE and CRP, older age and less years of formal education are the factors increasing the likelihood of having MCI in elderly patients with type 2 diabetes.

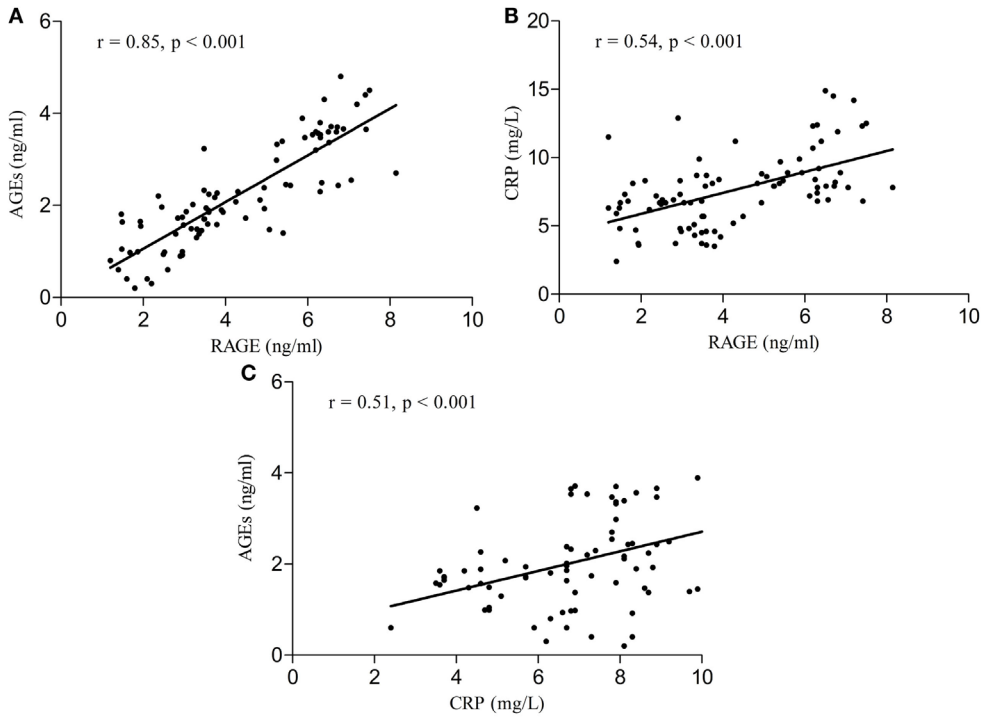

FIGURE 1 | (A) Correlation of RAGE with AGEs in group of diabetic elderly patients with MCI. **(B)** Correlation of RAGE with CRP in group of diabetic elderly patients with MCI. **(C)** Correlation of AGEs with CRP in group of diabetic elderly patients with MCI.

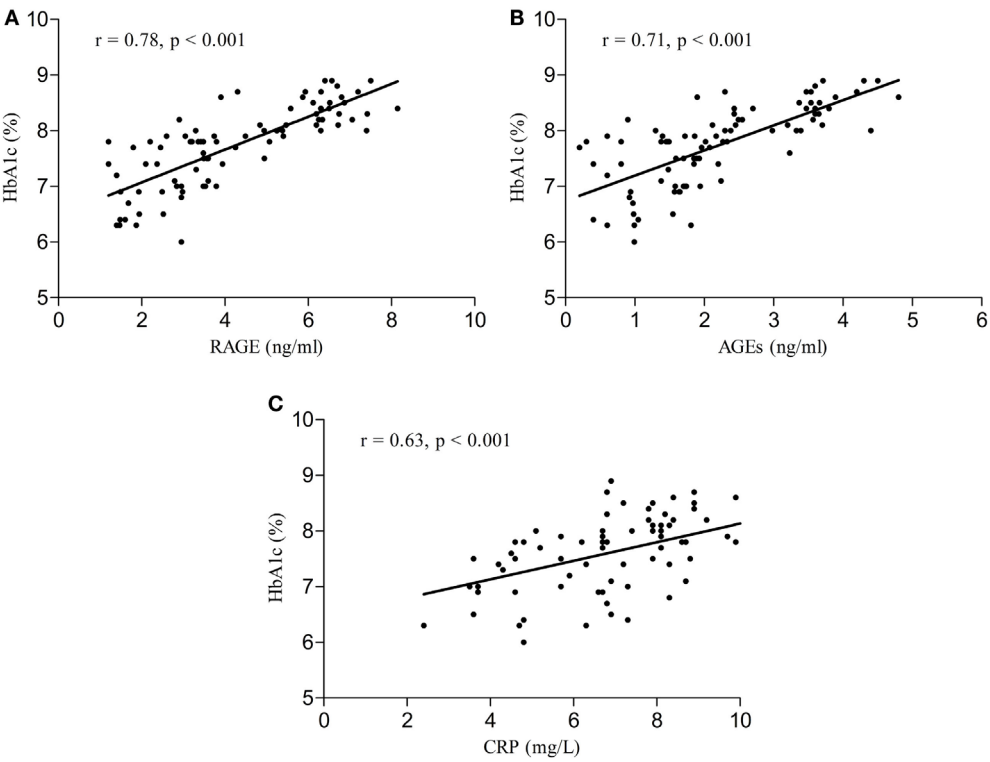

FIGURE 2 | (A) Correlation of HbA1c with RAGE in group of diabetic elderly patients with MCI. **(B)** Correlation of HbA1c with AGEs in group of diabetic elderly patients with MCI. **(C)** Correlation of HbA1c with CRP in group of diabetic elderly patients with MCI.

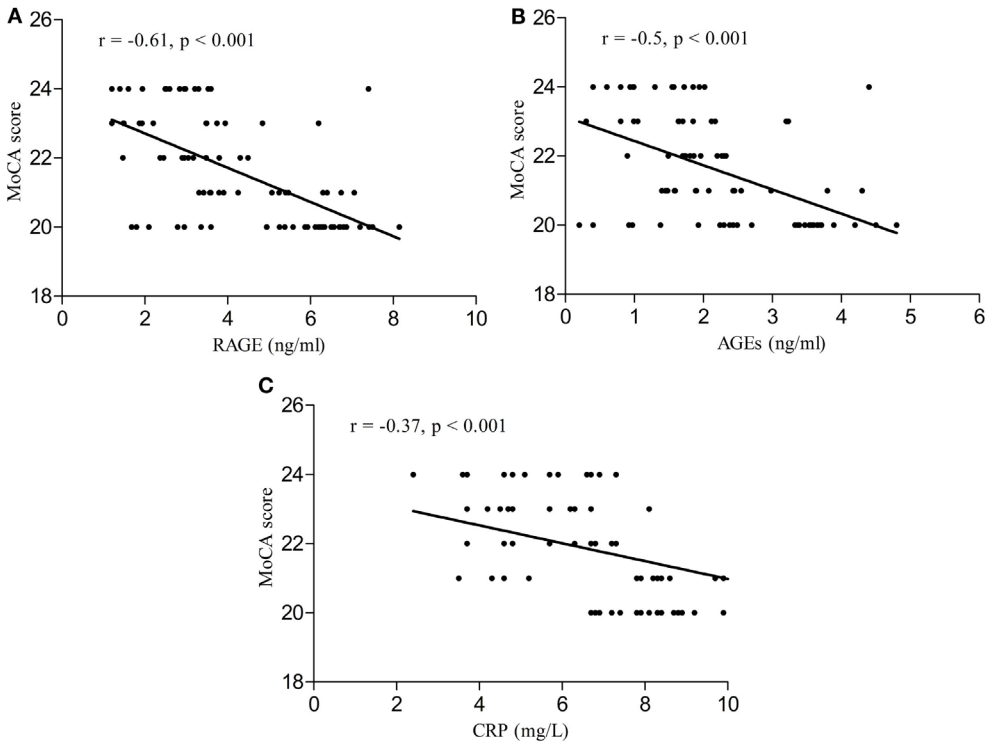

FIGURE 3 | (A) Correlation of MoCA score with RAGE in group of diabetic elderly patients with MCI. **(B)** Correlation of MoCA score with AGEs in group of diabetic elderly patients with MCI. **(C)** Correlation of MoCA score with CRP in group of diabetic elderly patients with MCI.

TABLE 3 | Relationship of serum levels of AGEs, RAGE, and CRP with other clinical indicators in group of diabetic elderly patients with MCI.

	AGEs	*p*	RAGE	*p*	CRP	*p*
	r		*r*		*r*	
MoCA score	–0.5	$p < 0.001$	–0.61	$p < 0.001$	–0.37	$p < 0.001$
HbA1c (%)	0.71	$p < 0.001$	0.78	$p < 0.001$	0.63	$p < 0.001$
Serum cholesterol (mmol/l)	0.1	0.33	0.12	0.25	0.35	0.001
Serum LDL-C (mmol/l)	0.06	0.6	0.08	0.43	0.27	0.01
Serum triglycerides (mmol/l)	0.26	0.014	0.37	$p < 0.001$	0.28	0.007
Serum HDL-C (mmol/l)	–0.2	0.056	–0.33	0.002	–0.19	0.06
AGEs (ng/ml)	1		0.85	$p < 0.001$	0.51	$p < 0.001$
RAGE (ng/ml)			1		0.54	$p < 0.001$
CRP (mg/l)					1	

**Significance, $p < 0.05$; r, correlation coefficient.*

BMI, body mass index; CHOL, total cholesterol; CRP, C-reactive protein; AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end products; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MoCA, Montreal Cognitive Assessment.

DISCUSSION

Our results show that patients with MCI had significantly higher CRP levels than controls. The increased CRP level is a predictor of cognitive impairment in elderly patients with type 2 diabetes. This result is consistent with recent reports. CRP – an acute-phase protein – is commonly used marker, which participates in low-grade inflammation in diabetes. It is mainly associated with the risk of DM complications. Other researchers had found serum levels of CRP were associated with cognition impairment and inversely correlated with MMSE and MoCA scores (Ge et al., 2013). In other studies, low-grade inflammation as reflected by serum CRP concentrations was found in subjects with cognitive dysfunction without dementia and in patients with non-amnestic MCI (Haan et al., 2008; Roberts et al., 2009). CRP is also associated with increased risk of CVD (Ridker et al., 1998). In our study, CRP as well as CVD is independent predictors of MCI in diabetic patients. Thus, the mechanism of the involvement of low-grade inflammation in cognitive impairment is not explained entirely by the presence of CVD in diabetic patients. CRP could initiate

TABLE 4 | Assessment results of the risk of having MCI in a simple logistic regression model in elderly patients with type 2 diabetes.

Variables analyzed	β	SE of β	*p*-value	OR	95% CI
Age (years)*	0.137	0.03	$p < 0.001$	1.15	1.08–1.22
Gender: female	0.2	0.1	0.12	1.2	0.49–1.59
Education (years)*	−0.639	0.09	$p < 0.001$	0.53	0.44–0.63
Smoked tobacco regularly	0.12	0.1	0.36	0.8	0.6–1.16
Duration of T2DM (years)*	0.097	0.02	$p < 0.001$	1.1	1.05–1.15
Previous stroke	0.41	0.27	0.14	1.5	0.87–2.58
Previous CVD*	1.43	0.16	$p < 0.001$	4.19	3.03–5.81
Previous HA or use of HA drugs*	0.88	0.22	$p < 0.001$	2.41	1.55–3.76
Hyperlipidemia*	0.72	0.21	0.001	2.01	1.35–3.12
Retinopathy*	0.8	0.14	$p < 0.001$	2.24	1.7–2.96
Nephropathy*	0.44	0.13	0.001	1.56	1.2–2.03
Neuropathy	0.11	0.01	0.45	1.12	0.82–1.53
Co-morbidity (*n*)*	0.426	0.05	$p < 0.001$	1.53	1.37–1.71
Depressive syndrome	0.03	0.01	0.8	0.96	0.73–1.27
BMI (kg/m^2)	0.058	0.03	0.1	1.06	0.98–1.13
HbA1c (%)*	1.69	0.23	$p < 0.001$	5.47	3.45–8.67
Serum cholesterol (mmol/l)	0.01	0.003	0.95	1.01	0.99–1.01
Serum LDL-C (mmol/l)	0.01	0.004	0.74	1.01	0.99–1.01
Serum triglycerides (mmol/l)*	0.02	0.004	$p < 0.001$	1.02	1.01–1.02
Serum HDL-C (mmol/l)*	−0.09	0.018	$p < 0.001$	0.91	0.87–0.94
CRP (mg/L)*	0.64	0.08	$p < 0.001$	1.9	1.62–2.22
AGEs (ng/ml)*	1.15	0.16	$p < 0.001$	3.17	2.31–4.34
RAGE (ng/ml)*	1.19	0.16	$p < 0.001$	3.28	2.42–4.45

**Significance, $p < 0.05$.*

β, regression coefficient; CI, confidence interval for odds ratio; OR, odds ratio; SE, standard error; T2DM, diabetes type 2; CVD, cardiovascular disease; HA, hypertension; BMI, body mass index; CHOL, total cholesterol; CRP, C-reactive protein; AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end products; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

TABLE 5 | Assessment results of the risk of having MCI in a multivariable logistic regression model in elderly patients with type 2 diabetes.

Variables analyzed	β	SE of β	*p*-value	OR	95% CI
Age (years)*	0.11	0.05	0.039	1.11	1.0–1.23
Education (years)*	−0.45	0.12	$p < 0.001$	0.64	0.5–0.82
Previous CVD*	1.2	0.24	$p < 0.001$	3.34	2.06–5.41
Previous HA or use of HA drugs*	1.24	0.41	0.002	3.48	1.56–7.76
CRP (mg/l)*	0.44	0.13	0.001	1.55	1.21–1.99
RAGE (ng/ml)*	0.67	0.23	0.004	1.95	1.24–3.06

**Significance, $p < 0.05$.*

β, regression coefficient; CI, confidence interval for odds ratio; OR, odds ratio; SE, standard error; CVD, cardiovascular disease; HA, hypertension; CRP, C-reactive protein; RAGE, receptor for advanced glycation end products.

immune cascade reactions leading to neurodegeneration via activating the complement system (Eagan et al., 2012).

Like CRP, AGEs–RAGE system stimulated by hyperglycemia could be responsible for the deterioration of cognition through an immune inflammatory pathway. AGEs – the products of non-enzymatic glycation on lysine and arginine residues accumulate in diabetic tissues – plays the important role in pathogenesis of diabetic complications. AGEs binds their receptor – RAGE and modulates cellular properties. Stimulation of RAGE results in activation of NADPH oxidase and generation of oxidation stress. Reactive oxygen species activate also formation of AGEs. In that way, the vicious circle is created – hyperglycemia induce AGEs generation, then after binding to RAGE increase oxidation stress and further production of AGEs (Kislinger et al., 1999; Wautier et al., 2001). Expression of RAGE is increased in CVD and in diabetes.

The frequency of cardiac events is higher and the progression of atherosclerosis is bigger in patients with diabetes compared to age-matched healthy controls. A lot of studies showed elevation of RAGE in diabetes and its association with diabetic complications and severity of the disease (Matsunaga-Irie et al., 2004; Yan et al., 2009). There are also data about increasing levels of AGEs with age and it is further enhancement by hyperglycemic state (Ulrich and Cerami, 2001). The interaction between AGEs and RAGE could result in higher inflammation in vascular wall via stimulation of oxidative stress. Activation of endothelial, smooth muscle cells and mononuclear phagocytes is highly associated with diabetic complications, such as nephropathy, retinopathy, and atherosclerosis (Schmidt et al., 2000b; Win et al., 2012).

Stimulation of RAGE play crucial role in the pathogenesis of T2DM and its complications, but RAGE is also an important cell-signaling receptor involved cognitive impairment. RAGE expression was found in different structures of the brain, in microglia, and astrocytes. In Alzheimer disease, RAGE expression was increased in inferior frontal cortex and hippocampus. The stimulation of RAGE in neurons can lead to increased inflammation through activation of the transcription faction NF-κB. In diabetes neuroinflammation, vascular injury and also activation of microglia could be driven by RAGE and the cascade of chemokines and cytokines (Lue et al., 2012).

In our study, AGEs and RAGE levels were significantly elevated in MCI subjects. The results are consistent with one study, which reported also higher level of AGEs in MCI subject with T2DM (Chen et al., 2011). The authors proposed possible the mechanism by which AGEs induce MCI in patients with type 2 diabetes. It is possible that AGEs stimulate endothelial dysfunction and followed the increased permeability of blood vessels. Thus, AGEs can accumulates in vascular wall and cause its injury. Other explanation could be higher inflammation and neural damage via inducing oxidative stress mediated by AGEs–RAGE interactions. AGEs could also activate microglia and destroy microtubular structure resulting in dysfunction of neurons (Chen et al., 2011).

In opposite to this result recent study was evaluated whether plasma levels of RAGE are altered in MCI and Alzheimer's disease. The authors did not found higher levels of RAGE in MCI but the study was performed among only 24 patients without vascular disease (only 16% of them had diabetes) (Marksteiner et al., 2014).

Expectedly, we found significant relationships between AGEs, RAGE, CRP, and glycosylated hemoglobin. Subjects with MCI had also more micro complications. Sustained hyperglycemia and development of diabetes complications can induce chronic

low-grade inflammation and cell activation via AGEs–RAGE interaction. In agreement with our results, other studies reported that persistent hyperglycemia, indicated by elevated HbA1c, is an independent risk factor for the cognitive dysfunction (Ryan and Geckle, 2000).

In our previous work, we found that the presence of depressive syndrome is associated with higher levels of inflammatory markers in elderly patients with diabetes (Gorska-Ciebiada et al., 2015). Therefore, we put this parameter into logistic regression models; however, we revealed that the presence of depressive syndrome have no influence on the results.

Limitations

This study provides important insights into AGEs–RAGE system disturbances and low-grade inflammation pathologies underlying cognitive impairment in older diabetic patients; however, it is not without limitations. First, because our study population was relatively small, our results should be interpreted with caution. Second, the study was not designed as longitudinal prospective investigation. There have been few studies in which the cognitive declines in elderly diabetics were prospectively observed. However, the precise mechanisms underlying T2DM-related cognitive dysfunction or the development of dementia have not yet been elucidated. Furthermore, this investigation was limited to patients with diabetes, and therefore, an association between AGEs, RAGE, and CRP with other parameters in subjects without diabetes should also be assessed.

CONCLUSION

In summary, serum AGEs, RAGE, and CRP are increased in the circulation of MCI elderly diabetic patients compared to controls. Higher level of RAGE and CRP, HA or use of HA drugs, previous CVD, older age and less years of formal education are the factors increasing the likelihood of having MCI in elderly patients with type 2 diabetes. The precise mechanisms responsible for this finding are not entirely clear. A larger population-based prospective study needs to be performed to further confirm the relationship between AGEs, RAGE, and the cognitive decline or progress to dementia. As an option, targeting the AGEs–RAGE system in diabetes especially with cognitive impairments through specific pharmacologic interventions might result in a clinical benefit for these patients.

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REVIEW
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Astrocytic estrogen receptors and impaired neurotrophic responses in a rat model of perimenopause

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In a perimenopausal model of middle-aged rats, the astrocyte estrogen receptor-alpha (ERa): ER-beta (ERb) ratio increased with the onset of acyclicity (constant estrus, CE) in association with impaired neurotrophic responses to estradiol (E2). We report additional data on irregular cycling (IR) from this study of 9 month old perimenopausal subgroups. In particular, irregular cyclers (IR) also show increased ERa:ERb ratio in cerebral cortex astrocytes comparable to acyclic individuals in CE. In mixed glial cultures from these same cycling subgroups, the E2-dependent neurotrophic activity and glial fibrillary acidic protein (GFAP) repression by E2 were impaired in IR to the same degree as in CE-derived glia. The greater importance of cycling status than age during the perimenopause to astrocyte ERs are attributable to individual variations of the residual ovarian follicle pool, which determine the onset of acyclicity. The corresponding loss of E2-dependent GFAP repression and E2-dependent neurotrophic activity add further to the inverse relationship of GFAP expression and astrocyte neurotrophic activity across aging in both sexes. These findings are relevant to impairments of spatial learning and of hippocampal long-term potentiation during the onset of IR in middle-aged rats, and to perimenopausal factors mediating the higher risk of women for Alzheimer disease.

Keywords: estrogen receptors, perimenopause, astrocytes, glial fibrillary acid protein, neurotrophic

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Introduction

Estrogen receptor-alpha (ERa) and estrogen receptor-beta (ERb) undergo age-related shifts in both sexes (Wilson et al., 2002; Wu et al., 2009; Arimoto et al., 2011, 2013; Arimoto, 2012; Foster, 2012). In brain, ER aging changes vary by cell type and hormonal status. In a perimenopausal model of middle-aged rats, the astrocyte ERa:ERb ratio was increased in acyclic constant estrus (CE) rats (Arimoto et al., 2013). The ERa:ERb ratio has broad significance to synaptic plasticity (Foster, 2012; Bean et al., 2014), which we have characterized for astrocytic neurotrophic support (Rozovsky et al., 2002, 2005; Arimoto et al., 2011, 2013). In particular, elevations of ERa:ERb decrease neurotrophic support, which is indirectly linked to expression of glial fibrillary acidic protein (GFAP), the astrocytic intermediate filament through its estrogen response elements (Rozovsky et al., 2002; Stone et al., 1998). The elevated ERa:ERb in astrocyte cultures from aging males was manipulated by RNAi, which decreased GFAP (Rozovsky et al., 2005; Arimoto et al., 2013). Both E2 responses (GFAP repression and induction of neurotrophic activity) are lost during aging (Rozovsky et al., 2005; Arimoto et al., 2013). In this review, we present data on irregular cycling (IR; Arimoto et al., 2011) that extends findings of Arimoto et al. (2013) and discuss how changes in astrocytic ERs during the perimenopause transition

may result from changes in steroids from both the ovary and the brain. The importance of IR status was shown in impaired learning of IR rats vs. RC (regular cycling) of the same age (Paris et al., 2011) and impaired long-term potentiation in hippocampal slices (Yin et al., 2015).

A group of 9 month old rats were characterized for cycling status: regular cycles 4–5 day (RC); irregular cycles >5 day (IR); and acyclic CE. The IR group was not reported in Arimoto et al. (2013) but was described in Arimoto et al. (2011) and the Ph.D. Thesis of Jason Arimoto (USC Department of Biological Sciences, 2012). First we describe astrocyte ER *in vivo* expression in 9 month old rats. Then we describe primary glial cultures from the same groups for astrocyte GFAP expression in relation to neurotrophic activity.

Astrocyte ERa:ERb Ratio Increases with Age

Age changes in astrocyte estrogen receptors (ER) are under-represented in the growing literature on ERs in brain aging, which has mainly focused on neuronal ERs (Bean et al., 2014; Kermath et al., 2014). Both sexes of aging rats (9–24 month, middle-age to old age) show increased ERa:ERb in astrocytes of cerebral cortex as an outcome of increased ERa and decreased ERb, as determined from immunohistochemical analysis of co-labeling for GFAP, the astrocyte-specific protein (Arimoto et al., 2013). **Figure 1** shows progressive increase of ERa and decrease of ERb during transitions from RC to IR to CE (**Figures 1A,B**). In the prior comparison of CE with RC (Arimoto et al., 2013), ERb levels were lower by 40%, whereas IR were just 10% lower (**Figure 1B**). Correspondingly, the ERa:ERb ratio increased by 1.2-fold in IR and 2.5-fold in CE (**Figure 1C**). In an older cohort at 13 month, CE and IR had similar shifts of ERa:ERb (Arimoto et al., 2013, not shown). Primary glial cultures of enriched astrocytes or mixed glial (astrocytes: microglia, 3:1) retain these age changes. Perimenopausal rats age 9 month show further complexity in comparisons of regular cyclers (RC) vs. irregular cyclers (IR) vs. acyclic CE. The data on IR (Arimoto et al., 2011; Arimoto, 2012) show ERa:ERb shifts *in vivo* that are comparable to the CE in 9 month old rats (**Figure 1A**). We suggest that astrocytes may be major contributors to the decrease of ERb in whole hippocampal RNA from IR vs. RC rats (Yin et al., 2015).

Inverse Relationships between GFAP and Neurotrophic Activity

Primary cultures of mixed glia (astrocytes:microglia, 3:1) were prepared from the other hemicortex from these rats (Arimoto et al., 2013). Mixed glia were used rather than enriched astrocytes because we wished to avoid artifactual perturbation of gene activity associated with hydrodynamic effects of shaking to remove microglia (Gatson et al., 2011). In male derived glia, mixed glia and enriched astrocytes showed the same age trends in ERs and GFAP, and neurotrophic activity (Arimoto et al., 2013). In glia from 9 month rats, astrocyte GFAP was repressed by E2 in RC, whereas IR and CE were unresponsive to E2 (**Figure 1D**). Correspondingly, neurotrophic activity in conditioned media assayed by neurite outgrowth was increased by E2 only in glia from regular cyclers (**Figure 1E**). The reciprocal relationship between GFAP expression and astrocyte neurotrophic activity (RC vs. IR vs. CE) is consistent with prior analysis, updated in **Figure 1F**.

Enhanced GFAP expression influences neurite outgrowth through astrocyte-secreted laminin and other extracellular factors (Rozovsky et al., 2002). Loss of E2-sensitivity of neurite outgrowth in male derived astrocytes was reversed by down regulation of GFAP by RNAi; conversely, young astrocytes acquired an aging phenotype by transfection with GFAP cDNA (Rozovsky et al., 2005). Because GFAP is regulated by E2 through a classical promoter ERE that binds ERa (Stone et al., 1998), we examined the role of ERs. The elevated ERa:ERb in astrocyte cultures from aging males was manipulated by RNAi to ERa, which decreased GFAP (Arimoto et al., 2013). However, neurotrophic activity was not restored, suggesting additional ER dependent mechanisms. The loss of E2 responses in GFAP repression and in E2-dependent neurotrophic activity may be the earliest impairment in gene responses in a rodent model.

Perimenopausal Transition

The rodent perimenopausal model (**Figures 2A,B**) is described with reference to the STRAW stages of human menopause (Finch, 2014). Rodents and humans undergo similar ovarian senescence, beginning with increasingly IR and declining fertility, and ending with total depletion of ovarian follicles (Finch et al., 1984). The CE following IR with modest sustained blood E2 and very low progesterone (P4) may be considered as a model for hyperestrogenic cycles during human peri-menopause (Finch, 2014). We hypothesize that the perimenopause may be characterized by a general trend for increasing plasma E2:P4 during the perimenopause (Finch et al., 1984; Finch, 2014), equivalent to progressive exposure to unopposed estrogen.

To study perimenopausal changes associated with cycle lengthening, we used a mixed glia model (3:1 astrocytes; microglia), which had shown the same age changes of ERa, GFAP, and neurotrophic activity as enriched astrocytes in males (Arimoto et al., 2013). Mixed glia from irregularly cycling 9 month rats and CE rats both show impaired E2-responses of GFAP and of neurotrophic activity. Thus, the loss of hypothalamic GFAP responses to E2 in middle-aged rats (Anderson et al., 2002) has a counterpart in cerebral cortex astrocytes. Moreover, it shows the impact of modest steroidal perturbations associated with irregular cycles to synaptic functions related to memory. In IR vs. RC of the same age, hippocampal slices show impaired LTP (Yin et al., 2015), while intact rats show impaired spatial learning (Paris et al., 2011). In an astrocyte-neuron

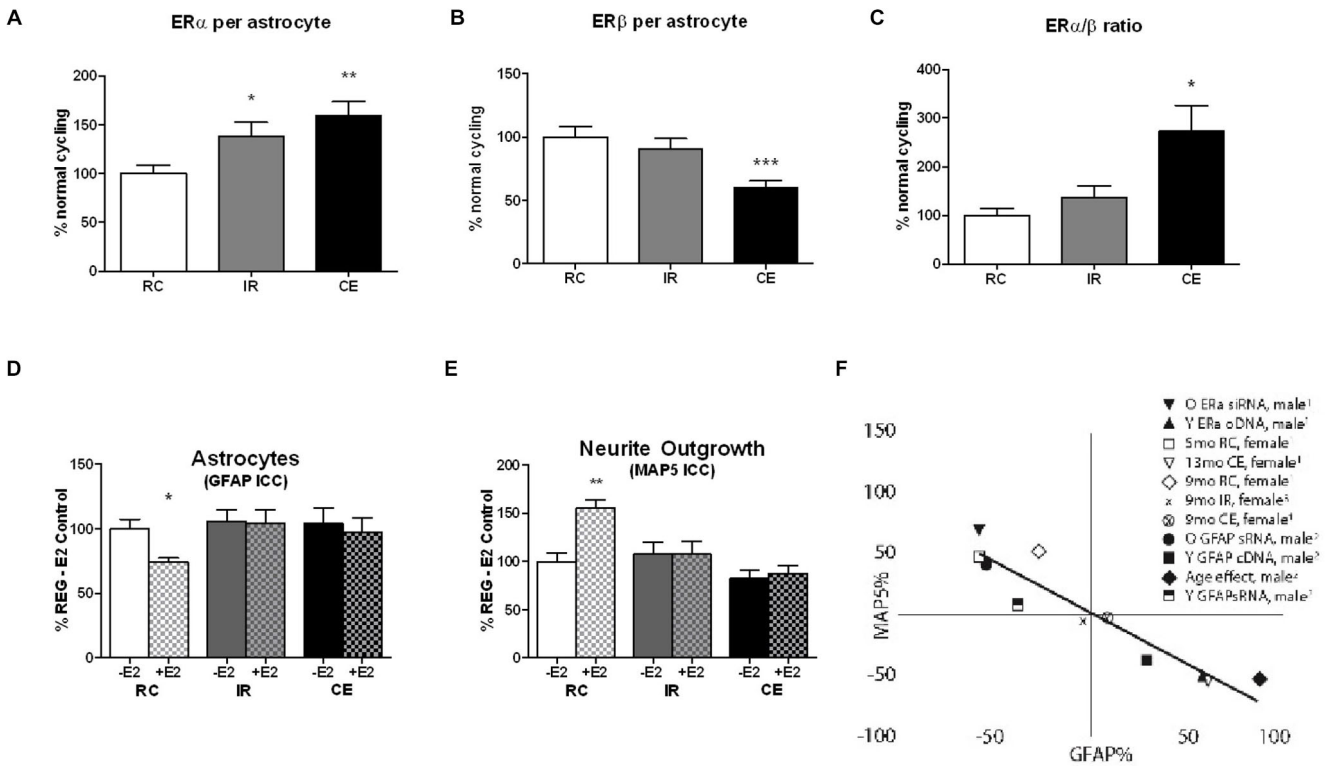

FIGURE 1 | (A–C) *In vivo* ERα and ERβ expression in cerebral cortex astrocytes of perimenopausal female rats, aged 9 month differ by ovarian cycle status: regular cycles (RC) 4–5 day irregular cycles (IR) >5 day; constant estrus (CE) (Nelson et al., 1982; Finch, 2014). Astrocyte ERs in primary visual cortex in layers 1 and 2/3, RC vs. IR vs. CE; 4 brains per cycle status; Data is expressed as % regularly cycling (RC) control; mean+SEM; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. RC and CE data published in Arimoto et al. (2013), Figure 7 (n.b. CE is denoted as AC, ibid.). The IR data are from Arimoto et al. (2011) and the thesis of Jason Arimoto (USC, 2012). **(D,E)** *In vitro* GFAP expression and neurotrophic activity (MAP5, neurite outgrowth) in mixed glia from 9 month old perimenopausal rats of different cycling status: RC vs. IR vs. CE. Mixed glial were co-cultured with E18 neurons; 4 cultures, 4 brains per cycling stage group. Data expressed as % RC control; mean + SEM; $^{**}p < 0.01$; $^{*}p < 0.05$. GFAP responses to 100 pM E2 in mixed glia from 9 month rats: The RC responded to E2 with decreased GFAP, but age-matched IR and CE were unresponsive. Neurite outgrowth (MAP5) was increased by 100 pM E2 in RC, but not in age-matched IR and CE. The RC and CE data were published in Arimoto et al. (2013), Figure 6 (n.b. CE is denoted as AC, ibid.). The IR data are from Arimoto et al. (2011) and the thesis of Jason Arimoto (USC, 2012). **(F)** Reciprocal relationships between GFAP and neurite outgrowth (MAP5), plotted as % change relative to controls in that experiment. RC and CE data published in Arimoto et al. (2013), Figure 8. New data on 9 month IR are calculated from **(D,E)**.

culture model, ERa was specifically associated with E2-induced glutamatergic synaptogenesis (Jelks et al., 2007). We suggest that the impairments of E2-dependent neurotrophic activity in IR rats can be used to identify E2-dependent neurotrophic factors underlying LTP and spatial memory.

Ovarian Contributions

We suggest that the early onset of ER changes in females is driven by ovarian steroidal changes. In particular, as estrous cycles undergo lengthening and transition to acyclicity, there is a modest reduction of the blood estradiol: progesterone ratio (E2:P4) (Finch et al., 1984). At much later ages, typically after 20 month in rodents, ovarian follicles are depleted with reductions of plasma E2 to ovariectomized (OVX) levels (Gee et al., 1983). Although male rodents do not undergo total reproductive senescence with the complete loss of fertility and gonadal steroids in older age, plasma testosterone tends to decrease in middle-aged male rats (Rosario et al., 2009).

Further perspectives come from studies of 3 decades ago in our lab. By 12–14 month, rodent ovaries have < 10% of oocytes and primary follicles remaining from the young adult (**Figure 2C**; Gosden et al., 1983). Note the wide range of oocyte numbers; this 5-fold wide range is hypothesized to underlie the individual differences in onset of acyclicity (Finch and Kirkwood, 2000).

The role of oocyte depletion in cycle lengthening was shown with ovarian transplants between young and middle-aged mice (heterochronic transplants; **Figure 2D**; Felicio et al., 1986). Replacing young ovaries with middle-aged ovaries caused premature cycles lengthening to >5 days. Despite the wide range of remaining follicles in middle-aged ovaries (**Figure 2C**), the remaining number is close to the threshold required for ovulatory cycles. The tight linkage of cycle lengthening to the

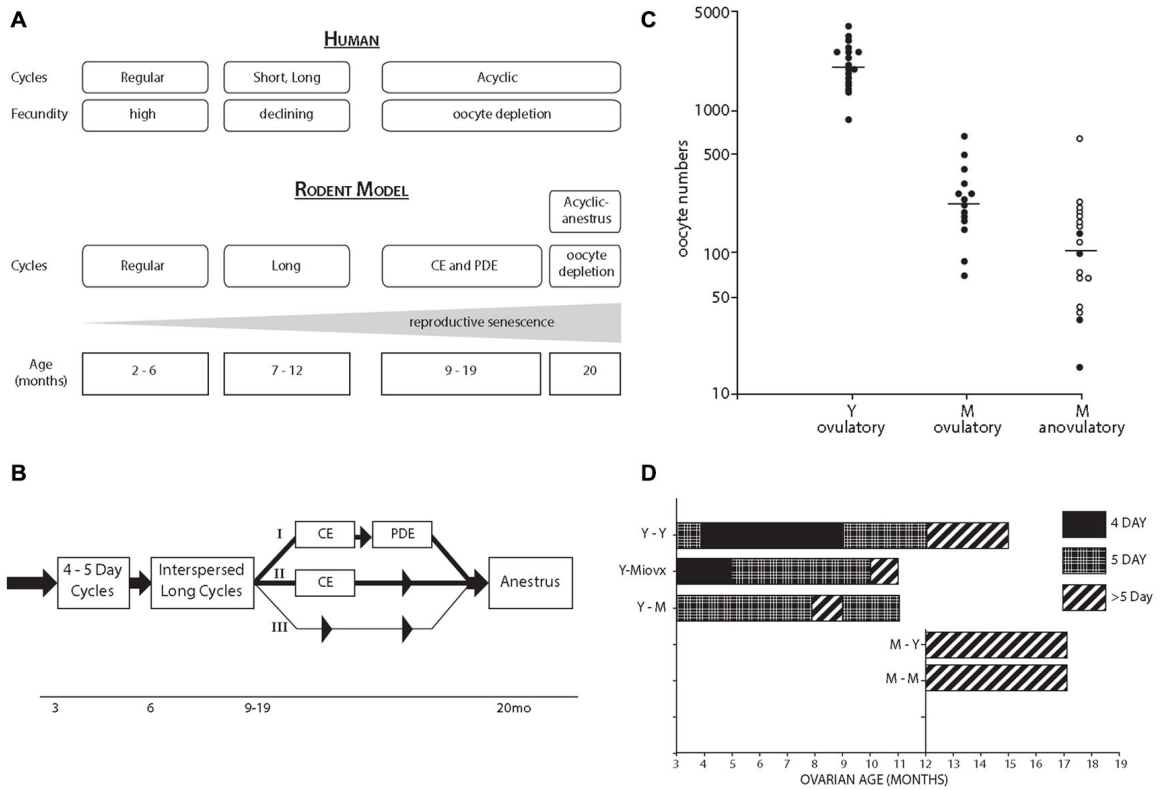

FIGURE 2 | (A) Comparison of perimenopausal transitions in women and rodents (lab mouse and rat). The most common initial acyclic state of rodents, CE is driven by continued ovarian estradiol (E2) production at low levels with hypothalamic impairments of the E2-dependent preovulatory gonadotrophin surge. Alternate pathways with persistent diestrus (PDE) are shown in panel **B**. The terminal state of anestrus with total oocyte depletion arise after 20 month, with LH elevations as in postmenopausal women. Rodent perimenopause occupies a larger fraction of the 28 month life span than for women, spanning the onset of cycle irregularity after 6 month to ovarian follicle depletion after 20 month, which comprises half the lifespan. For details, see Nelson et al., 1982; Felicio et al., 1984; Finch et al., 1984; Gee et al., 1983; Finch, 2014. **(B)** Rodent perimenopausal transitions with alternate trajectories from lengthening cycles interspersed with 4–5 day ovulatory cycles (I) CE followed by PDF (repetitive pseudopregnancy), and thence to anestrus; (II) CE directly to anestrus; (III) Lengthening cycles leading directly to anestrus (least common). Adapted from Finch et al. (1984), **(C)** Studies on C57BL/6J mice from the Finch lab. Ovarian oocyte numbers in Y (young, 4–5 month) and M (middle-aged, 13–14 month); the M were grouped by the occurrence of recent ovulation. Horizontal lines are median values. From Gosden et al. (1983). **(D)** Heterochronic ovary transplantation between Y and M ages. The MLovx were ovariectomized (OVX) at 3 month and given ovaries at 12 month; their aging without exposure to ovarian steroids maintained the potential for 4 day cycling, not seen in Y-M transplant. Redrawn from Felicio et al. (1986).

remaining ovarian follicles is also shown by the induction of premature cycle lengthening and CE by surgically removing 90% of young ovarian mass (Nelson and Felicio, 1986).

Neuroendocrine Contributions

Neuroendocrine mechanisms are also at work, as shown by transplant of young ovaries to middle-aged mice, which yielded mostly 5 day cycles. However, if middle-aged mice were OVX when young and allowed to age without the presence of ovaries (long-term ovariectomy, Lt-OVX), then 4 day cycles were observed. Thus, the loss of 4 day cycles has a neuroendocrine component that is modified by estrogen exposure. The estrogen exposure hypothesis of cycle lengthening was tested by several durations of exposure to exogenous E2, which advanced the onset of acyclicity, in which the neuroendocrine component was shown by transplantation of control ovaries (Mobbs et al., 1985; Kohama et al., 1989b). As few as 6 weeks of low sustained E2 accelerated the onset of acyclicity (Kohama et al., 1989b). The importance of the E2:P4 ratio was shown by the protective effect of P4 implants (Kohama et al., 1989a). We attribute these effects of E2:P4 on synaptic remodeling rather than neurodegeneration because chronic E2 did not change the number of hypothalamic LHRH or TIDA neurons (Kohama et al., 1992). Astrocyte GFAP is a mediator of estrous cycles by the close relation of GFAP-containing astrocyte processes to the LHRH neurons which shift during proestrus (Garcia-Segura et al., 1994). The E2-dependent induction of hypothalamic GFAP was impaired in middle-aged rats in parallel with the loss of the E2-induced LH surge (Anderson et al., 2002). Subsequently, we developed *in vitro* systems for studying GFAP regulation that further documents impaired GFAP responsiveness with functional connections to neuronal plasticity.

Conclusion

Laboratory rodents show a perimenopausal transition with lengthening cycles that has both brain (neuroendocrine) and ovarian contributions, as shown by prior ovarian transplantation studies between different age groups. Recent data further show that the neurotrophic activity of astrocytes assayed *in vitro* declines in the earliest perimenopausal stage of cycle irregularity and with cycle lengthening. These findings are relevant to impairments of spatial learning and of hippocampal long-term potentiation during the onset of IR and to perimenopausal factors mediating the higher risk of women for Alzheimer disease.

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REVIEW
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Interaction of basal forebrain cholinergic neurons with the glucocorticoid system in stress regulation and cognitive impairment

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A substantial number of studies on basal forebrain (BF) cholinergic neurons (BFCN) have provided compelling evidence for their role in the etiology of stress, cognitive aging, Alzheimer's disease (AD), and other neurodegenerative diseases. BFCN project to a broad range of cortical sites and limbic structures, including the hippocampus, and are involved in stress and cognition. In particular, the hippocampus, the primary target tissue of the glucocorticoid stress hormones, is associated with cognitive function in tandem with hypothalamic-pituitary-adrenal (HPA) axis modulation. The present review summarizes glucocorticoid and HPA axis research to date in an effort to establish the manner in which stress affects the release of acetylcholine (ACh), glucocorticoids, and their receptor in the context of cognitive processes. We attempt to provide the molecular interactive link between the glucocorticoids and cholinergic system that contributes to BFCN degeneration in stress-induced acceleration of cognitive decline in aging and AD. We also discuss the importance of animal models in facilitating such studies for pharmacological use, to which could help decipher disease states and propose leads for pharmacological intervention.

Keywords: cholinergic neuron, stress, aging, basal forebrain, hippocampus, glucocorticoid, glucocorticoid receptor

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Introduction

Since its inception, the cholinergic hypothesis has generated considerable interest. It has been used to decipher the different orchestrating functions and dysfunctions in the nervous system associated with Alzheimer's disease (AD) and other neurodegenerative diseases. This hypothesis has been used to further understand cognitive impairment and neurodegenerative diseases by evaluating and tracing brain functions in normal and aging brains (Gallagher and Colombo, 1995; Contestabile, 2011). The main components of the cholinergic pathway are: (1) the neurotransmitter acetylcholine (ACh); (2) acetylcholinesterase (AChE), which breaks down ACh; (3) choline acetyltransferase, an enzyme that synthesizes ACh; and (4) ACh receptors, specifically the nicotinic ACh receptor, and the muscarinic ACh receptor (mAChR). Evidence from previous research on normal aging (Drachman et al., 1982),

AD (Whitehouse et al., 1982), and anti-cholinergic (Newhouse et al., 1988, 1994) and pro-cholinergic drug administration (Davis and Mohs, 1982) supports the major role of the cholinergic system in aged-related cognitive decline. Extensive research has established the relationship between cognitive impairment and the cholinergic system in the basal forebrain (BF; Baxter and Chiba, 1999). The involvement of the cholinergic system in regulating stress is also evident from studies that acute/inescapable stress enhanced release of ACh and induced expression of genes that regulate ACh availability in the hippocampus and prefrontal cortex (Mark et al., 1996; Kaufer et al., 1998). Cognitive processes are influenced by the acute and chronic stress-induced release of glucocorticoids, stress hormones that influence the function of the prefrontal cortex and hippocampus (Popoli et al., 2011). Stress and stress hormone plays a well-established role in mental health and impaired cognition. It is correlated with hippocampal volume and age-related cognitive decline (Lupien et al., 1994, 2009), suggesting that sustained stress, via glucocorticoid hypersecretion, leads to hippocampal damage (Uno et al., 1989). Prolonged overproduction of glucocorticoids can be detrimental to brain structure, whereas insufficient glucocorticoid signaling can lead to stress-related pathological conditions (Raison and Miller, 2003). This emphasizes the need for the careful regulation of glucocorticoid exposure. Impairment of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress is also associated with cognitive dysfunction in aged animals (Issa et al., 1990; Bizon et al., 2001). In addition, HPA activity was blunt in elderly compared to young adult participants (Hatzinger et al., 2011). Therefore, new therapeutic approaches acting on the HPA axis and its receptor signaling should take into account.

Activation of the septo-hippocampal cholinergic system is considered as an important aspect in the adaptive response to stress and is influenced by neuronal and hormonal stimuli. This septo-hippocampal activation seems to initialize following activation of the pituitary-adrenocortical axis (Gilad et al., 1985; Gilad, 1987) and may then affect glucocorticoid secretion via the HPA axis (Herman et al., 1996). The HPA axis plays an important role in the adaptation to stress by modulating hippocampal activity. Thus, the hippocampus, along with cholinergic innervation from the BF, is involved in regulating the HPA axis stress response. Activation of the HPA axis mediates responses that enable an organism to maintain its homeostasis. Hence, neurodegeneration of cholinergic neurons, a pathological characteristic in AD and aging, makes the elderly vulnerable to stress, resulting in cognitive impairment.

The extent to which the cholinergic system is involved in stress, cognition, and neurological disorders has been reported in several significant research reports. Therefore, we attempt to develop a supporting background for our recent studies in rats (aged or with cholinergic lesions) on HPA axis dysfunction in response to stress and altered glucocorticoid receptor (GR) signaling in the hippocampus. We also describe the interactive link between the glucocorticoid and cholinergic systems in aging and stress.

Overview of the Basal Forebrain Cholinergic System

An anatomical simplified overview of BF cholinergic neurons is summarized in **Figure 1**, including cells located in the medial septum (MS), the vertical limb of the diagonal band of Broca (VDB), and the nucleus basalis of Meynert–extending to the substantia innominata in the rodent brain. These structures send cholinergic projections to a broad range of neocortical sites as well as structures in the limbic system, including the hippocampus (Mesulam et al., 1983; Gallagher and Colombo, 1995). Thus, the septo-hippocampal pathway, which arises from the medial septal nucleus and nucleus of the diagonal band, is the main structure of the central cholinergic system and the main source of cholinergic innervation to the hippocampal formation. A topographical model of the septo-hippocampal pathway has been constructed based on various lesion, tracing, and immunocytochemical methods in the septal and hippocampal regions (Lewis et al., 1967; Dutar et al., 1995). The MS is connected to the hippocampus, via the fimbria and dorsal fornix, and to the medial cortex (Lewis and Shute, 1967; Teles-Grilo Ruivo and Mellor, 2013). The cornu ammonis (CA) 1 pyramidal and dentate granule (DG) cell layers in the dorsal hippocampus receive afferent inputs from the VDB, and these cell layers in the ventral hippocampus receive inputs from the both MS and VDB (McKinney et al., 1983; Nyakas et al., 1987).

Cholinergic Hypothesis in Disease Etiology

The cholinergic hypothesis has been implicated in the etiology of AD, various types of dementia, and aging, and is rooted in degeneration of BF cholinergic neurons causing cognitive deficit (Bartus et al., 1982; Bartus, 2000; Sarter et al., 2003). This theory, however, remains controversial. Although enhancement of cholinergic function by cholinergic agents (e.g., AChE inhibitors) in AD and age-related cognitive deficit supported the hypothesis, other research subsequently pinpointed the involvement of other factors, such as dopamine projections to the frontal cortex, amyloid deposition, and increased glucocorticoid levels (Dumas and Newhouse, 2011). More specifically, hypersecretion of glucocorticoid, or A-beta-altered HPA axis function, have been implicated in hippocampal impairment in AD (Hibberd et al., 2000; Brureau et al., 2013) Additionally, elevated glucocorticoid levels and impaired GR signaling are associated with HPA dysfunction, resulting in cognitive decline in elderly subjects (Issa et al., 1990; Lupien et al., 1994; Bizon et al., 2001; Mizoguchi et al., 2009). Therefore, a possible pathophysiological link between glucocorticoids and the age-dependent decline in BF cholinergic function, especially in the CA1, CA3, and DG regions of hippocampus, has also been established (Hörtnagl et al., 1993). Craig et al. postulated a new cholinergic hypothesis version for AD, where loss of MS cholinergic input to the hippocampus induces hippocampal vulnerability, resulting in greater cognitive impairment in response to subsequent insults, such as stress or injury (Craig et al., 2011).

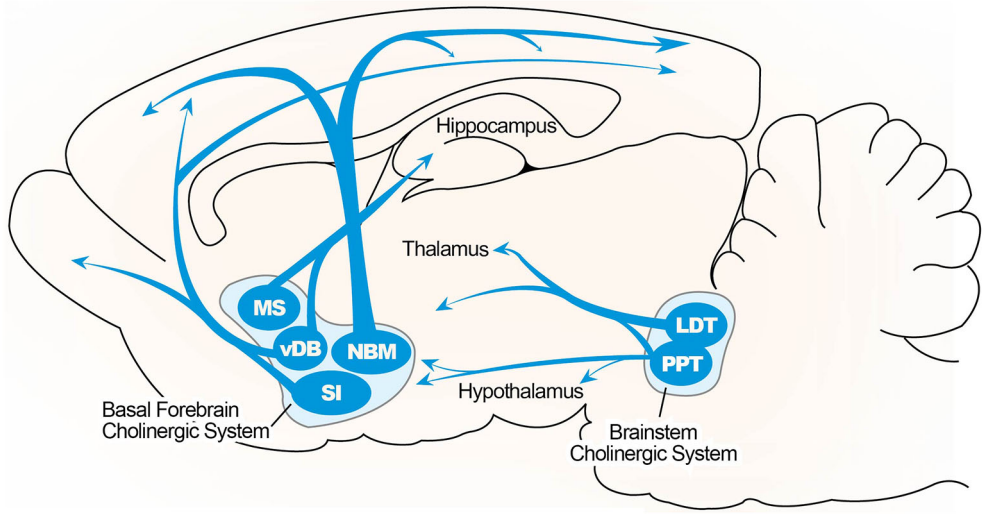

FIGURE 1 | Illustrated overview of the basal forebrain cholinergic pathway. Cholinergic projections include the medial septum (MS), vertical limbs of the diagonal band of Broca (vDB), nucleus basalis of Meynert (NBM), and substantia innominate (SI) projecting to the hippocampus, thalamus, olfactory bulb, and cortical region. Cholinergic pontomesencephalon neurons include laterodorsal tegmental (LDT) and pedunculopontine tegmental nuclei (PPT) projecting to hindbrain, thalamus, hypothalamus, and basal forebrain.

Implication of Lesion Studies in Evaluating Cholinergic Innervation

Application of the cholinergic hypothesis to animal model offers the ability to evaluate functional network and molecular pathway to understand neurocognitive diseases. Selection of animal (even among rodents) is important, as they differ in cholinergic tone, receptor activation, and relevance to human basal cholinergic activity (Van der Zee and Keijser, 2011). The adoption of the rodent as an animal model may compliment studies in primates due to effectiveness and cost-efficiency. Moreover, features such as short lifespans are an important factor to be considered in the study of late-onset or aging diseases (Gallagher et al., 2011). One major pathological hallmark of neurodegenerative disease that cause cognitive decline, is the dramatic loss of BF cholinergic projection neurons with reduced cholinergic innervation to the hippocampus and neocortex (Davies and Maloney, 1976; Whitehouse et al., 1981; Arendt et al., 1983; Mesulam, 2004). Additionally, the relative loss of cholinergic neurons and the decrease of the ACh synthesizing enzyme, choline acetyltransferase in the brain of AD patients is associated with cognitive impairment (Bartus, 2000). In animal studies, neurotoxin-induced BF lesions cause similar cognitive impairments (Olton, 1990). However, lesioning cholinergic BF neurons is challenging because they are intertwined with non-cholinergic neurons and there is a risk of damaging adjacent structures.

Although BF lesions using conventional lesion methods (e.g., electrolytic) produce varying manifestations of cognitive impairment, the development of methods that selectively interrupt the BF region has been attempted (Easton et al., 2012; Baxter and Bucci, 2013). An initial approach innovatively used 192IgG-saporin, a neurotoxin with a specific affinity for cholinergic neuron cell surface receptors (Wiley et al., 1991; Baxter and Bucci, 2013). A myriad of studies picked the hippocampal and septo-hippocampal regions of the BF as lesion sites to evaluate the cholinergic interventions observed in aging and cognition. Studies with selective BF neurotoxic lesion concluded this region was associated with cognitive function characterized by attention (Muir et al., 1993; Baxter et al., 1997, 1999; Bucci et al., 1998; Chiba et al., 1999; Chudasama et al., 2004), and learning and memory (Hepler et al., 1985; Hagan et al., 1988; Berger-Sweeney et al., 1994; Baxter et al., 1995; Janisiewicz et al., 2004). Furthermore, a significant correlation between cognitive impairment and decline in cholinergic markers for the septo-hippocampal projection in aged rats (Gallagher et al., 1990; Smith and Booze, 1995) supports the involvement of BF cholinergic neurons in cognition. More recently, animals with a saporin-induced partial loss of septo-hippocampal cholinergic neurons exhibited cognitive deficit (Brayda-Bruno et al., 2013). Other lesion studies extended the importance of the BF cholinergic system in the process of functional recovery from brain injury in young rats (Conner et al., 2005), which is intriguing in that animals with BF lesions may show deficits in cognitive function. Selective lesion of cholinergic inputs to the hippocampus has also been used to evaluate the effects of cholinergic receptors in regulating hippocampal ACh release (Thorne and Potter, 1995).

Though lesion studies have generated substantial controversy, the advent of this approach has generated new empirical tests and compelling information on the potential function of cholinergic and non-cholinergic neurons emanating from the BF. Additionally, this has unveiled the possible role played by loss of these neurons in stress, aging, and other neurocognitive diseases. More essentially, animal models are indispensable for recreating specific human pathogenic events, and invaluable for drug screening and therapeutic intervention assessment.

HPA Axis and Glucocorticoids in Stress Regulation and Aging

The HPA axis is an integral component in promoting resilience to stress. Most salient age-related changes in the stress response are thought to occur in the HPA axis. The HPA system is classically controlled by a range of afferent signals responsible for the coordinated release of various signaling markers. The hypothalamus is at the top of the hierarchy in the control of the central HPA axis. When the brain perceives a stressor, activation of the paraventricular nucleus of the hypothalamus triggers the release of corticotropin-releasing hormone. Corticotropin-releasing hormone stimulates pituitary adrenocorticotropic hormone (ACTH) release and activates the adrenal gland, which secretes glucocorticoids (cortisol in humans and corticosterone in rodents). Glucocorticoids released from the adrenal gland interact with the HPA axis by binding to specific GRs in the brain, forming a closed-loop feedback system and subsequently coping with stress (Sapolsky et al., 1986; Mizuno and Kimura, 1997; Srinivasan et al., 2013). Thus, glucocorticoids are the end product of the HPA axis and regulate a wide array of actions influencing neuronal function and metabolism (**Figure 2**).

Two types of steroid hormone receptors have been identified in tandem in the brain: the mineralocorticoid receptor (MR) and the GR. Expression of MR is considerably more restricted in the brain compared to the ubiquitous expression of GR, and both mediate classical genomic and non-genomic glucocorticoid actions by acting as nuclear transcriptional activators and repressors (Reul and de Kloet, 1985; Joëls, 2008; van der Laan and Meijer, 2008; Groeneweg et al., 2011). Moreover, the secretion of glucocorticoids and their binding to receptors leads to auto-regulatory decreases in receptor availability and *vice versa* (Sapolsky et al., 1984; Reul et al., 1987a,b). Actions of GR described above extends the role of GR beyond mediating glucocorticoid feedback following stress, whereas MR participates in basal HPA tone (Reul et al., 1987a; De Kloet et al., 1998) and the genomic action in adaptation and homeostasis after stress exposure (de Kloet, 2008). After GR binding, these activated receptors are translocated to the nucleus where they bind to the glucocorticoid responsive elements (GRE) and affect the transcriptional activity of target genes (Funder, 1997). Stress causes a down-regulation of GR through the increase of circulating glucocorticoids, eventually decreasing sensitivity to nuclear transcriptional activities. Glucocorticoid-GR can also activate transcription by binding directly as a homodimer to the GRE DNA sequence present in the promotors of target genes (e.g., *serum- and glucocorticoid-induced protein kinase, mitogen-activated protein kinase phosphatase-1*, etc) that has been reported to regulate cognitive function (Lee et al., 2007; Vyas and Maatouk, 2013; Cestari et al., 2014).

HPA activity tends to increase with age due to inefficiencies of glucocorticoid negative-feedback inhibition, resulting in elevated plasma levels of ACTH and glucocorticoids (Mizoguchi et al., 2009; Aguilera, 2011). A decrease in glucocorticoid negative-feedback inhibition is associated with the loss of GR and altered GR signaling in the forebrain (Issa et al., 1990; Bizon et al., 2001; Lund et al., 2004; Lee et al., 2012). Increased levels of glucocorticoids appear to threaten hippocampal neurons and direct the loss of dendrite complexity in the hippocampus (Hibberd et al., 2000). Inappropriate HPA axis regulation in aging is reviewed by Aguilera (2011) who stresses its adverse effect in stress-related brain disorders.

A link between HPA deregulation and disruption of GR has been observed in AD and other neurodegenerative diseases (Brureau et al., 2013; Vyas and Maatouk, 2013). Interestingly, though extra-hypothalamic GR sites receive attention for this glucocorticoid-mediated HPA inhibitory feedback; the hippocampus receives the utmost attention with regards to these effects (McEwen et al., 1968, 1969; Sapolsky et al., 1984, 1986; Herman et al., 1989). The influence of glucocorticoids on the HPA axis markedly depends on the available GR in individual

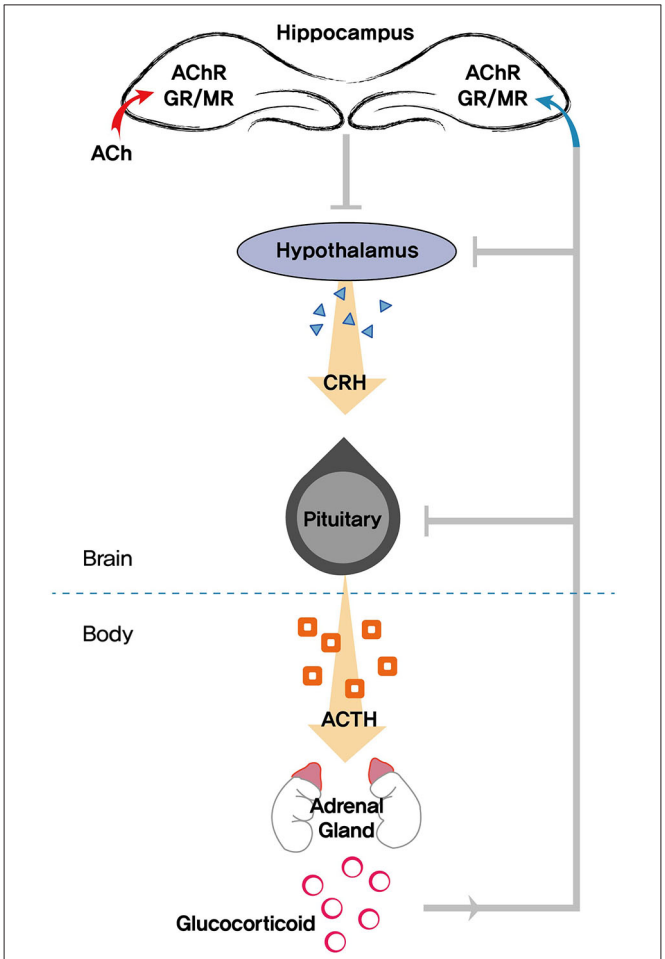

FIGURE 2 | Schematic diagram showing the role of the hippocampus in modulating the HPA (hypothalamic pituitary adrenocortical) axis. Stress triggers the release of glucocorticoids, which exerts feedback to the hippocampus, hypothalamus, and pituitary. Acetylcholine (ACh) is also involved in mediating neuroendocrine, emotional, and physiological responses in tandem with the HPA axis. AChR, acetylcholine receptors; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone.

tissues (Simons, 2008). Abundant expression of GR in the forebrain (McEwen et al., 1969; McEwen and Wallach, 1973), and increased activity of the HPA axis in the absence of forebrain inhibition on HPA axis by damage of the forebrain (Jacobson and Sapolsky, 1991; van Haarst et al., 1996), contributes to the sequelae associated with GR malfunction. Taken together, forebrain GR expression is critical for HPA axis regulation in response to stress (Furay et al., 2008) and further adduces the role of GR in stress.

Cholinergic Neuron in Stress Regulation via HPA Axis and Cognitive Function

A further increase in ACh release was observed in the hippocampus after acute stress (Finkelstein et al., 1985; Gilad et al., 1985; Imperato et al., 1989). Corticosterone administration, mimicking the increase in the plasma corticosterone concentration produced by stress, induced hippocampal ACh (Imperato et al., 1989). These studies support the involvement of glucocorticoids in the cholinergic innervation of the hippocampus, and the activation of the HPA axis in the process. Stress-induced responses activated the septo-hippocampal cholinergic pathway within minutes (Gilad, 1987), which then induced ACh-mediated neuroendocrine, emotional, and physiological responses by stimulating the HPA axis (Newman et al., 2001). This HPA axis activation led to the release of corticosterone, a stress neurohormone (Nyakas et al., 1987; Calogero et al., 1988, 1990). Increased release of hippocampal ACh and glucocorticoids in response to stress was observed in young rats, but not in aged rats (Mizuno and Kimura, 1997). However, contradictory results have been reported. Proteomic analyses of the hippocampus of rats exposed to stress showed a decrease in a precursor protein of hippocampal cholinergic neurostimulating peptide (HCNP) leading to a loss of ACh production (Kim and Kim, 2007). HCNP stimulate the enzyme activity of choline acetyltransferase in neurons. It is also reported that expression levels of HCNP precursor protein mRNA were decreased in the hippocampus of AD patients (Maki et al., 2002). These reports indicate a possible intricate interplay between the level of glucocorticoids, ACh, and hippocampal cholinergic protein expression effecting septo-hippocampal cholinergic pathways.

Interestingly, a parallel increase in both plasma corticosterone and hippocampal ACh level has been validated as a consequence of elevated platform exposure, a relatively mild stress (Degroot et al., 2004). The interaction of glucocorticoid with ACh in the brain is well reviewed (Mora et al., 2012). Moreover, regulation of the HPA axis by glucocorticoid feedback and cholinergic brain function modulating stress responses depends on the intensity and predictability of stressful stimuli (Pitman et al., 1988; Martí and Armario, 1997; Morris and Rao, 2014).

Although ACh mediates its effects via both types of ACh receptors, mAChR are more involved in cognitive impairment and are densely present in the hippocampus (Dutar et al., 1995; Colgin et al., 2003; Drever et al., 2011). An excessive loss of mAChR in the hippocampus of Alzheimer's patients, and severely impaired muscarinic signaling associated with age-related cognitive decline (Bartus et al., 1982; Zhang et al., 2007), reveal the connection between the muscarinic-dependent cholinergic system and cognitive impairment. Scopolamine-induced mAChR blockade resulted in cognitive deficit in healthy adult humans (Voss et al., 2010). Additionally, mAChR antagonists significantly elevated plasma corticosterone in stressed rats, suggesting an inhibitory effect of mAChR stimulation on pituitary-adrenal function (Kile and Turner, 1985). This result is indicative of a correlation between corticosterone levels, mAChR availability, and cognitive function.

Basal Forebrain Cholinergic Neurons, Hippocampal Glucocorticoids, and Glucocorticoid Receptor in Stress Regulation and Cognitive Aging

The hippocampus is a brain structure crucially involved in memory, the neuroendocrine regulation of stress hormones, and termination of the stress response via HPA axis glucocorticoid-mediated inhibition (Mizuno and Kimura, 1997; Kim and Diamond, 2002). Studies showing hippocampal damage due to prolonged exposure to glucocorticoids or chronic stress in primates (Uno et al., 1989; Sapolsky et al., 1990) have been triggered an assessment of the cumulative impact of such exposures on the hippocampus using other animals. A number of studies reported that chronic stress or glucocorticoids contributed hippocampal cell death in adult rats (Sapolsky et al., 1985; Dachir et al., 1997). The hippocampus contains a high density of GR and is a target of glucocorticoid actions. Cognitive deficits are associated with a loss of hippocampal neurons, in particular pyramidal cells, due to increased glucocorticoid exposure (McEwen et al., 1968; McEwen, 1999; Hibberd et al., 2000). However, others failed to find hippocampal neuronal loss in rats (Bodnoff et al., 1995; Sousa et al., 1998; Coburn-Litvak et al., 2004), tree shrews (Vollmann-Honsdorf et al., 1997; Fuchs et al., 2001), primates (Leverenz et al., 1999), and humans (Müller et al., 2001). The inconsistencies in literatures on glucocorticoid-related cell death may arise from species-specific differences in expression levels of GR and MR (Conrad, 2008).

In any case, impairments of hippocampal dependent memory and synaptic plasticity, and structural alterations have been observed in the animals with chronic stress or corticosterone treatment (Bodnoff et al., 1995; Fuchs et al., 2001; Finsterwald and Alberini, 2014). Down-regulation of GR in the hippocampus follows the chronic corticosterone treatment. (Tornello et al., 1982). And GR signaling was altered in stress-related psychopathologies (Finsterwald and Alberini, 2014). Thus this indicates a connection between GR availability and cognitive function. As GR are responsible for negative feedback control of the adaptive stress response (De Kloet et al., 1998), a reduction in hippocampal GR is associated with post-stress glucocorticoid hypersecretion (Sapolsky, 1996). Additionally, glucocorticoid treatments exacerbated the cholinergic neurotoxin ethycholine aziridinium (AF64A)-induced cholinergic lesions in the hippocampus, suggesting

a pathophysiological link between glucocorticoids and age-dependent declines in cholinergic function or cholinergic degeneration in AD (Hörtnagl et al., 1993). Moreover, the essential coordinating role of GR in regulating glucocorticoid secretion through the HPA axis in response to stress in aging is well documented (Issa et al., 1990; Herman et al., 1996; Bizon et al., 2001; Murphy et al., 2002; Furay et al., 2008; Mizoguchi et al., 2009).

Glucocorticoid receptor, when functioning as a ligand-dependent transcription factor, controls transcription by directly binding to positive and negative GRE, regulating transcriptional increases (anti-inflammatory) or decreases (HPA axis negative feedback) or inhibiting transcriptional activity of other factors (on pro-inflammatory molecules) (Silverman and Sternberg, 2012). The GR-DNA binding phenomenon is gradually receiving recognition, and research on the anti-inflammatory effects of GR-DNA binding has been reported in *in vivo* and *in vitro* studies (Reichardt et al., 1998, 2001; Schäcke et al., 2002; Clark, 2007). Target disruption of GR genes and impaired GR-DNA binding has been correlated with cognitive deficits in mice (Oitzl et al., 1997, 2001), while intra-hippocampal GR blockade with a GR antagonist produced memory impairments (Nikzad et al., 2011). Diminished GR signaling and GR mRNA in the aged hippocampus is related to memory impairment and HPA axis dysregulation (Bizon et al., 2001; Murphy et al., 2002; Lee et al., 2012). Furthermore, a decrease in the nuclear uptake of corticosterone, decreased nuclear translocation, and DNA binding deficits were observed in the hippocampus of the aged rat (Sapolsky et al., 1983; Murphy et al., 2002; Lee et al., 2012). This highlights the need to understand glucocorticoids-genomic interactions, as this may illuminate the role of GR in cognitive processes. Reduced expression of GR mRNA in the hippocampus and medial prefrontal cortex was also observed with memory-impaired aged rats relative to young controls and memory-unimpaired aged rats, with no change in the basal levels of circulating glucocorticoids (Bizon et al., 2001). Another source of GR signaling interference in hippocampal cognition may be mediated by the regulation of other intruding nuclear transcriptional factors, such as activator protein and nuclear factor κB (NF-κB; Yang-Yen et al., 1990; McKay and Cidlowski, 1998; Lund et al., 2004). Recently, reduced expression of FKBP5, a key GR modulator, and smaller hippocampal volumes were observed in posttraumatic stress disorder, which was reversed after cognitive behavioral therapy (Levy-Gigi et al., 2013). Supporting the above facts, decreased nuclear GR mRNA and protein was observed in aged rats with cognitive impairment, suggesting defective GR transport might affect the transcriptional properties of hippocampal neurons with HPA axis dysfunction and could have age-related impact on cognitive decline and the loss of stress regulation (Bizon et al., 2001; Lee et al., 2012).

Although high levels of glucocorticoids are not associated with the loss of hippocampal neurons (Leverenz et al., 1999), some studies suggest interplay between the accumulative factor of stress and aging in the process of cell loss (Hibberd et al., 2000). On the other hand, Notarianni recently proposed a role for GR signaling in the initiation and development of AD, implicating over-activation of GR with hypercortisolemia in promoting amyloid beta (Aβ) production that leads to Aβ deposition and associated neuroinflammation (Notarianni, 2013).

Chronic neuroinflammation in the BF is also linked to loss of cholinergic neurons and is responsible for cognitive impairment associated with aging and AD (Willard et al., 1999). Significant loss of cholinergic neurons in the MS/diagonal band was also observed in aged animals with memory impairment (Baskerville et al., 2006). A direct correlation between glucocorticoid regulation of GR via the HPA axis and impaired GR function as a mechanism for inflammation is well reviewed (Silverman and Sternberg, 2012), emphasizing its importance in the prevention and management of chronic stress. Further, upregulation of pro-inflammatory cytokines occurs in the cortex and hippocampus of rats with post-surgery stress, resulting in post-operative cognitive dysfunction. This surgery-induced inflammation can be reduced by acetylcholinesterase inhibitors (Kalb et al., 2013), pointing to the involvement of the cholinergic system in cognitive impairment associated with neuroinflammation.

Interaction of ACh Receptor and GR in the Hippocampus

Cognitive impairment was observed in healthy human subjects treated with scopolamine, a selective mAChR antagonist (Voss et al., 2010). Rats treated with scopolamine showed spatial working memory impairment in an 8-arm radial maze task and alterations in ventral hippocampi ACh release (Mishima et al., 2000). In addition, impairment of recognition memory in BF cholinergic lesioned animals was aggravated by scopolamine, emphasizing the importance of mAChR in cognitive function (Steckler et al., 1995). Deficit in the transduction of cholinergic mAChR signals has been detected in the hippocampus of aged rats (Smith and Booze, 1995), the cortex of aged monkeys (Vannucchi and Goldman-Rakic, 1991), and in AD patients (Flynn et al., 1991). Additionally, impaired mAChR binding was found in the striatum and hippocampus of aged rats (Anson et al., 1992; Yamagami et al., 1992; Nieves-Martinez et al., 2012).

Loss of mAChR exacerbates cognitive decline and AD pathology, such as increased plaques/tangles and cerebrovascular deposition of Aβ in AD mice (Medeiros et al., 2011). Treatment with selective muscarinic agonists resulted in reduced production of Aβ in AD patients (Hock et al., 2003). A speculative pathway, due to loss of mAChR function in rats, induced by selective hippocampal cholinergic lesions with AF64A is predicted to influence effects in stimulating nicotinic receptors that may modulate the release of ACh (Thorne and Potter, 1995). In context, it is predicted that loss of mAChR function might exert stimulatory effects on nicotinic receptors, which are well described in cognitive functions (Levin, 2013). This highlights the importance of mAChR in BF cognitive function. Decreased expression of mAChR in the hippocampal CA1 region of aged epileptic animals (Cavarsan et al., 2011), and severely impaired muscarinic signaling in the hippocampus of cognitively impaired rats (Zhang et al., 2007), illustrates the involvement of the cholinergic system with cognition. More recently, decreases in ACh and mAChR were observed in cognitively impaired mice (Park et al., 2013). Additionally, impaired hippocampal ACh

release and cognitive deficits in mAChR knockout mice (Tzavara et al., 2003) coupled with mAChR antagonist impairment of memory in aged rats (Quirion et al., 1995; Klinkenberg and Blokland, 2010) implies a role for mAChR in the cholinergic hypothesis of cognition.

Activation of septo-hippocampal cholinergic neurons is manifested by increased release of ACh and choline uptake. This choline uptake is reduced below control levels in the presence of chronic stress, followed by an up-regulation of muscarinic binding sites (Finkelstein et al., 1985). Suppression of glucocorticoid secretion enhances hippocampal cholinergic transmission in rats (Mizoguchi et al., 2008). Furthermore, enhanced memory consolidation by striatal corticosterone injection was blocked by administration of scopolamine (Sánchez-Resendis et al., 2012), confirming the interactive relationship between glucocorticoids and cholinergic receptor. Glucocorticoid modulation of mAChR in lung (Scherrer et al., 1997), smooth muscle (Emala et al., 1997), chronic obstructive pulmonary disease (Johnson, 2005), and several brain nuclei in rats (Torres et al., 1991) suggests a similar pattern in hippocampal cholinergic neurons.

Rats with selective removal of hippocampal cholinergic input showed HPA axis dysfunction and decreased hippocampal GR levels (Han et al., 2002; Helm et al., 2002, 2004; Lim et al., 2012). Subsequent studies also revealed altered GR-protein kinase A (PKA)-NF-κB signaling in the hippocampus with loss of cholinergic input (Lim et al., 2011, 2012). The study regarding interactive effects of stress with loss of BFCN on cognitive function reported chronic stress induced impairment of working memory in rats with loss of hippocampal cholinergic input (Craig et al., 2008). Recently, we examined whether chronic stress aggravated cognitive deficit induced by selective BF cholinergic lesions leading to alterations in GR-PKA-NF-κB signaling. Lesioned rats receiving chronic stress showed a severe impairment in spatial memory and increased NF-κB signaling activation, which was substantiated by increased hippocampal pro-inflammatory gene expression, such as inducible nitric oxide synthase and cyclooxygenase-2 (Lee et al., 2013). These data indicate that the interaction between GR and ACh receptors is associated with stress-induced cognitive dysfunction.

Conclusion

The present review summarizes the current research and shows the importance of glucocorticoids and their receptor in modulating cognition in stress, aging, and AD via the cholinergic system. This may pave a new way in understanding the progression of the aforementioned diseases. Therefore, this review facilitates the understanding of the following: (1) involvement of both GR and ACh receptors in modulating cognition, thus providing a palliative approach for pharmaceutical interventions as these receptors are discussed in many research papers as a route to therapeutic intervention; (2) an interactive platform to mark the holistic consequences of cognitive impairment converging from varied neurological deficits; and finally; (3) an interactive role of glucocorticoids in the development of cognitive dysfunction and vulnerability of the hippocampus to such exposure. The main objective of this review was not only to highlight the possible underlying association between the various pathways and neural circuits involved in cognitive impairment, but also to enable the mitigation of such stress-induced cognitive morbidity by developing more effective pharmacotherapeutic strategies to ameliorate such diseases.

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Hematopoietic cytokines as therapeutic players in early stages Parkinson's disease

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Parkinson's disease (PD) is a devastating age related neurodegenerative disease that is believed to have a lengthy prodromal state. It is critical to find methods to harness compensatory recovery processes in order to slow or prevent the eventual progression of clinical symptoms. The current perspective paper argues that immune system signaling molecules represent such a promising therapeutic approach. Two cytokines of interest are granulocyte macrophage-colony stimulating factor (GM-CSF) and erythropoietin (EPO). These hematopoietic cytokines have been protective in models of stroke, neuronal injury, and more recently PD. It is our belief that these trophic cytokines can be used not only for cell protection but also regeneration. However, success is likely dependent on early intervention. This paper will outline our perspective on the development of novel trophic recovery treatments for PD. In particular, we present new data from our lab suggesting that EPO and GM-CSF can foster neural re-innervation in a "mild" or partial lesion PD model that could be envisioned as reflecting the early stages of the disease.

Keywords: Parkinson's disease, early stage, cytokines, granulocyte macrophage-colony stimulating factor, erythropoietin

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Parkinson's Disease: Animal Model of Early Stages

Parkinson's disease (PD) is characterized by a loss of dopamine (DA) neurons within the nigrostriatal pathway and the presence of Lewy body pathological protein aggregates (Farrer et al., 2001; Sherer et al., 2001). Clinically, PD is diagnosed based on tremors within distal limbs, muscle rigidity, and bradykinesia. By the time patients present with these motor symptoms, there has already been significant degeneration of DA neurons, with up to an 80% loss of striatal DA innervation (Bezard et al., 2001). There are also extensive non-motor symptoms, which present long before the cardinal motor symptoms (McDonald et al., 2003).

Current PD treatments only manage symptom severity and are not able to reverse or even appreciably slow the neurodegenerative processes. Thus, it is of interest to investigate potential treatments that could stabilize these surviving neurons and possibly induce some degree of neuronal recovery. It might be advantageous to target processes linked to the early or prodromal stages of PD, as neuronal plasticity would likely be more amenable to modulation at such times. However, models of early stage PD are less common and not as well understood as the late stage typically used. The neurotoxin, 6-hydroxydopamine (6-OHDA), is routinely used to induce PD-like pathology, inducing a loss of substantia

nigra pars compacta (SNc) DA neurons and downstream striatal terminals (Alvarez-Fischer et al., 2008). 6-OHDA infused directly into the SNc rapidly produces a robust degeneration of SNc DA neurons, coupled with striatal DA depletion within 48–72 h (Blandini et al., 2008; Thiele et al., 2012). However, this method has the obvious caveat of not reflecting the chronic slow course of degeneration. A more progressive lesion has been observed with lower doses of 6-OHDA infused into the striatum rather than SNc. Indeed, intra-striatal 6-OHDA administration induced a lesion, which gradually increased in size over several weeks (Sauer and Oertel, 1994) and more closely mimicked the progression from early to later stages of PD.

Novel Treatment Strategies

One exciting new avenue for treating PD involves the use of trophic factors to stabilize neuronal viability and even promote some degree of recovery. In fact, recent studies have revealed a reduction of brain derived neurotrophic factor (BDNF) within the SNc of PD patients (Mogi et al., 1999; Salehi and Mashayekhi, 2009). Accordingly, BDNF can promote the survival and differentiation of mesencephalic DA neurons, as well as protect against the DA toxicants, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-OHDA (Murer et al., 2001). Likewise, glial derived neurotrophic factor (GDNF), has also emerged as a potential candidate for neuroprotection in PD patients, based on success in various animal models (Fox et al., 2001; Ai et al., 2003). However, the improvements observed in clinical trials were restricted to the immediate area surrounding the site of infusion (Gill et al., 2003) and a randomized placebo-controlled study was unsuccessful at replicating these beneficial effects (Lang et al., 2006). Moreover, BDNF and GDNF do not readily cross the blood brain barrier (BBB) and have numerous side effects (Pezet and McMahon, 2006; Pilakka-Kanthikeel et al., 2013).

Finding well-tolerated factors with trophic properties, which cross the BBB represents a considerable challenge. Two cytokines that may hold potential therapeutic significance are the hematopoietic cytokines, erythropoietin (EPO) and granulocyte macrophage-colony stimulating factor (GM-CSF). Indeed, GM-CSF had protective effects in models of Alzheimer's disease (Boyd et al., 2010), and in MPTP and paraquat models of PD (Kim et al., 2009; Mangano et al., 2011). Moreover, GM-CSF administration induced spontaneous axonal regeneration and functional recovery from traumatic spinal cord injury (Ha et al., 2005; Bouhy et al., 2006) and reduced infarct volume following ischemia (Nakagawa et al., 2006; Schäbitz et al., 2008). Similarly, EPO has been investigated extensively for use in stroke, traumatic head injury and more recently, in toxin based animal models of PD (Sargin et al., 2010; Merelli et al., 2013; Bond and Rex, 2014). EPO was also shown to protect hippocampal neurons from stressor-induced apoptosis, and increased adult hippocampal neurogenesis (Merelli et al., 2015).

GM-CSF and EPO have well-documented trophic actions in the periphery and can infiltrate and accumulate within the brain (Enzler and Dranoff, 2003). Receptors for GM-CSF and EPO have been found on mature DA neurons and neural progenitor cells, suggesting that they might influence adult neuronal functioning, as well as stimulate maturation (Kim et al., 2004; Ha et al., 2005).

The peripheral function of GM-CSF is to promote the differentiation and maturation of innate immune cells, and it is routinely administered to cancer patients to modify neutrophil production (Dale et al., 1998). Similarly, EPO has potent mitogenic effects on immune cells, as well as red blood cells and is routinely prescribed for anemia and in the context of certain cancer treatments (Debeljak et al., 2014). Thus, both also have well established clinical track records.

GM-CSF and EPO Promote Striatal Re-Innervation

It is thought that a slow progressive "wave" of neurodegeneration occurs over many years before a critical threshold of neuronal loss is reached and clinical PD pathology is manifested. Accordingly, it is of interest to study the effects of potential treatments while the disease is still in an early or possibly even a prodromal stage. In the present paper, it was of interest to assess whether EPO or GM-CSF treatment could influence striatal innervation following the establishment of a partial or "mild" lesion that might be analogous to the early onset of the disease. To this end, male Sprague Dawley rats received a single intra-striatum (1.0 mm anterior, 3.0 mm lateral, 5.0 mm ventral relative to bregma) infusion of 6-OHDA (20 μg). Animals were then randomly divided into three groups (n = 7): Saline, EPO, and GM-CSF. On post-6-OHDA infusion Days 13 and 28, the animals received an intraperitoneal injection of either saline, recombinant human EPO solution (rhEPO; 50 μg/kg) or recombinant rat granulocyte-macrophage colony-stimulating factor solution (rat GM-CSF; 10 μg/kg). Animals were sacrificed on Day 30.

As shown in **Figure 1**, we did indeed find that a modest [∼10% of striatal area, as determined using tyrosine hydroxylase (TH) staining] lesion was induced in rats following intra-striatal infusion of a single moderate dose of 6-OHDA. However, no significant neuronal loss was evident within the SNc using this relatively mild paradigm, although it is possible that some of these SNc neurons would eventually die if we explored longer time intervals. Indeed, many surviving neurons had irregular shaped soma (**Figure 2**).

Importantly, the two EPO and GM-CSF injections that were given *after* the presumed establishment of the lesion (i.e., on Days 13 and 28 following 6-OHDA) provoked a significant recovery (presumed re-innervation) of striatal terminals. The statistical analysis revealed a significant treatment effect, $F_{(2,15)} = 10.7$, $P < 0.01$, with regards to striatal lesion size (**Figure 1**), such that the cytokines prevented the striatal lesion by Day 30 following 6-OHDA.

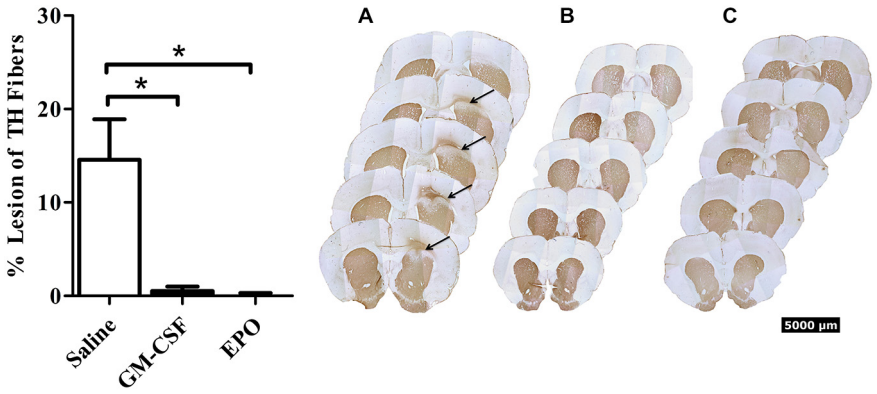

FIGURE 1 | Thirty days after the intra-striatal infusion of 6-OHDA, saline treated animals (graph—white bars; A) had a modest but statistically significant loss of TH+ striatal fibers. The GM-CSF (graph—black bars; **B**) and EPO (graph—black bars; **C**) treated animals displayed no visible lesion at the 30-day sacrifice time. Data is expressed as mean ± 1 SEM, $^*p < 0.01$.

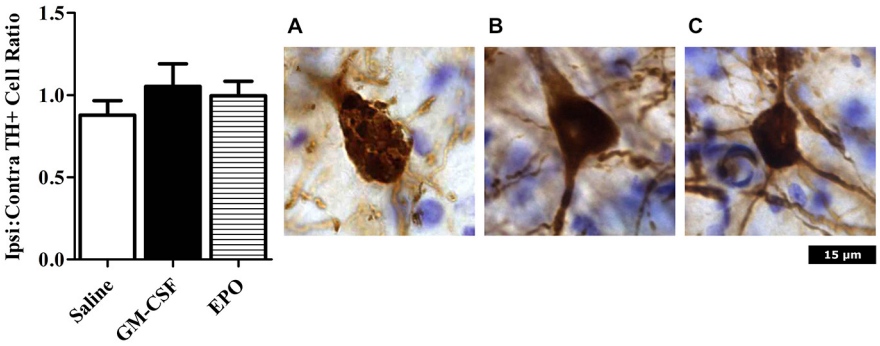

FIGURE 2 | Neurons were counted in an unbiased manner using MBF Stereo investigator optical fractionator probe. There were no significant differences between treatment groups (left graph). Photomicrographs are representative of animals treated with **(A)** Saline; **(B)** GM-CSF; or **(C)** EPO in conjunction with 6-OHDA infusion. The saline (but not GM-CSF or EPO) treated rats that received 6-OHDA displayed TH+ neurons with an abnormal shaped nucleus, with a reduction of projections. Images were taken at 40 × magnification.

The present findings are consistent with our own previous data and those of others showing beneficial effects of GM-CSF (Mangano et al., 2011; Kosloski et al., 2013) and EPO (Xue et al., 2007; Dhanushkodi et al., 2013; Qi et al., 2014) in toxicant animal models. However, since GM-CSF or EPO were administered after lesion establishment, the effects of the cytokine treatments in the current study would be expected to reflect some degree of recovery involving DA fiber re-growth rather than the prevention of fiber loss in the first place. This is a particularly novel finding given that the majority of studies typically focus on neuroprotective effects, rather than addressing the more clinically relevant issue of promoting recovery following some degree of neuronal damage.

Impact and Future Directions

It is important to underscore that the 6-OHDA paradigm presently used provoked a very modest loss of striatal terminals and future studies are required to ascertain whether GM-CSF and EPO might also have reparative properties in PD models with more significantly sized lesions. Nonetheless, GM-CSF and EPO may be ideal trophic treatment candidates based on their biological profiles, preclinical data and track record of clinical applicability. Mechanistically, these cytokines are potent inducers of BDNF and GDNF (Bouhy et al., 2006; Mengozzi et al., 2012), which we posit to be fundamental for their beneficial neural consequences. Targeting trophic processes to boost plasticity may be a critically important shift in treatment modalities away from failed attempts to translate neuroprotective approaches to the clinic; this strategy would also work well in tandem with recent efforts to identify biomarkers of disease state.

Author Contributions

CR and SH conceived the study. KF, CR, and SH designed the study. KF and CR performed the *in vivo* procedures. KF performed the immunohistochemical stains and data analysis. NP performed the stereological SNc counts. KF and SH prepared the manuscript. All authors have read and approved the final manuscript.

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REVIEW
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Protective effects of ginseng on neurological disorders

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Ginseng (Order: Apiales, Family: Araliaceae, Genus: *Panax*) has been used as a traditional herbal medicine for over 2000 years, and is recorded to have antianxiety, antidepressant and cognition enhancing properties. The protective effects of ginseng on neurological disorders are discussed in this review. Ginseng species and ginsenosides, and their intestinal metabolism and bioavailability are briefly introduced. This is followed by molecular mechanisms of effects of ginseng on the brain, including glutamatergic transmission, monoamine transmission, estrogen signaling, nitric oxide (NO) production, the Keap1/Nrf2 adaptive cellular stress pathway, neuronal survival, apoptosis, neural stem cells and neuroregeneration, microglia, astrocytes, oligodendrocytes and cerebral microvessels. The molecular mechanisms of the neuroprotective effects of ginseng in Alzheimer's disease (AD) including β-amyloid (Aβ) formation, tau hyperphosphorylation and oxidative stress, major depression, stroke, Parkinson's disease and multiple sclerosis are presented. It is hoped that this discussion will stimulate more studies on the use of ginseng in neurological disorders.

Keywords: ginseng, ginsenoside, neuroprotection, neurodegeneration, neurons, glial cells, brain

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Introduction

Ginseng (Order: Apiales, Family: Araliaceae, Genus: *Panax*) roots, stems, and leaves have been used as traditional herbal medicine for over 2000 years. In Korea, China and Japan, ginseng is the most valuable of all medicinal herbs. Its anti-anxiety, antidepressant and cognition-enhancing effects has been recorded by Shi-Zhen Li in Ben Cao Gang Mu (本草纲目) which is the most comprehensive pre-modern herbal text, compiled during the Ming Dynasty in China. *Panax ginseng* (人參) is mostly cultivated in Korea, the Manchuria region of China ("dong bei") and the coastal region of Siberia due to its sensitivity to temperature and soil conditions. *Panax quinquefolium* L (American ginseng, 西洋參) is cultivated in southern Canada and the USA, and *Panax notoginseng* (田七) is grown in the Yunnan and Guangxi provinces of China. These three herbs represent the most extensively investigated species of *Panax*. The latter means "cure all", and constituents of ginseng root produce adaptogenic, restorative, immunomodulatory, vasodilatory, anti-inflammatory, antioxidant, anti-aging, anticancer, anti-fatigue, anti-stress and anti-depressive effects in rodents and humans (Attele et al., 1999; Shin et al., 2000; Chang et al., 2003; Choo et al., 2003; Cheng et al., 2005; Wang et al., 2009). The bioactive ingredients in ginseng root include more than 60 ginsenosides, e.g., Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, Rg2, and Rg3, as well as polysaccharides, fatty acids, oligopeptides and polyacetylenic alcohols

(Qi et al., 2010). The purpose of this review is to discuss the effects of ginseng on the normal brain, and its protective effects in neurological disorders such as Alzheimer's disease (AD), major depression, stroke, Parkinson's disease and multiple sclerosis. It is hoped that will stimulate more studies on the use of ginseng in neurological disorders.

Intestinal Metabolism and Actions of Ginseng

Ginsenosides are triterpene saponins that have a common four ring hydrophobic steroid-like structure with sugar moieties (monomeric, dimeric, or trimeric) attached mainly at carbons 3, 6 and 20 (Zhu et al., 2004; Nah et al., 2007). Ginsenosides are divided into two different structural classes: the 20 (S)-protopanaxadiol (PD) group of ginsenosides Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd, Rg3 and Rh2 and the 20 (S)-protopanaxatriol (PT) group ginsenosides Re, Rf, Rg1, Rg2, and Rh1 (Baek et al., 2012). **Figure 1** shows the chemical structures of the two ginsenosides Rb1 and Rg1. In addition, several new ginsenosides such as 25-OH-pPD and 25-OH-pPT have been isolated and characterized from the fruit. 25-OH-pPD shows strong cancer preventing activity *in vitro* (Wang et al., 2007). Four malonyl derivatives of ginsenosides Rb1, Rb2, Rc and Rd have also been described (Fuzzati, 2004). The different sugar moieties in ginsenosides have been proposed to provide specificity for the therapeutic effects of ginsenosides (Zhu et al., 2004; Nah et al., 2007). The most commonly studied ginsenosides are Rb1, Rg1, Rg3, Re, and Rd. Intact ginsenosides are absorbed only from the intestines (the absorption rate is as low as 1 to 3.7%) and most ginsenosides are metabolized in the stomach (acid hydrolysis) and in the intestine (bacterial hydrolysis) or transformed to other ginsenosides. *In vitro* studies have indicated that ginsenoside Rb1 is metabolized by human intestinal bacteria. The metabolism is initiated by ginsenoside Rd (Rd) pathway at the C-20 glucose (Rb1 → Rd → ginsenoside F2 (F2) → Compound K (Cpd K) (Hasegawa et al., 1997; Hasegawa and Uchiyama, 1998; Tawab et al., 2003; Hasegawa, 2004; Bae et al., 2006; Qian et al., 2006; Chen et al., 2008b; Hou et al., 2012; Jung et al., 2012; Zhao et al., 2012). Collective evidence suggests that the *metabolism and transformation of intact ginsenosides* is an important process, playing an important role not only in bioavailability but also in the potential health benefits of ginseng (Chen et al., 2008a), such as anti-tumor (Xu et al., 2007; Lee et al., 2009), antioxidant, anti-inflammatory (Keum et al., 2003; Bae et al., 2006), anti-fatigue and angio-suppressive effects (Yue et al., 2006). Thus, ginseng is a natural remedy which not only enhances phagocytosis, natural killer cell activity, psychological function, cardiac function, and exercise performance, but also improves immune function by enhancing the production of interferons and increasing resistance to stress (Kiefer and Pantuso, 2003).

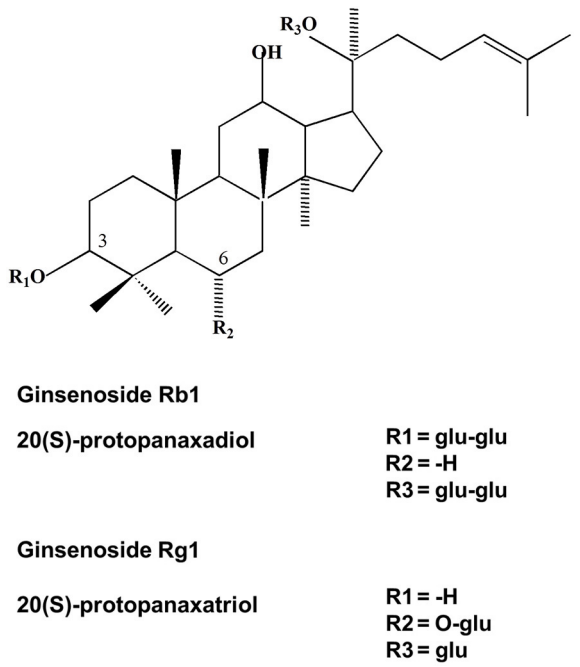

FIGURE 1 | Chemical structure of ginsenosides Rb1 and Rg1. Ginsenoside Rb1 is an example for 20 (S)-PD and ginsenoside Rg1 is an example for 20 (S)-PT type of ginsenosides.

Many of the beneficial effects of ginseng are mediated through modulation of enzymes involved in signal transduction processes (**Table 1**). Administration of ginseng extract over 10 days inhibited peroxisome proliferator-activated receptor-α function, and decreased the expression of several genes involved in lipid and lipoprotein metabolism, but increased serum concentrations of total cholesterol, high-density lipoprotein cholesterol and triglycerides (Yoon et al., 2003). Jiannaoning, a Chinese herbal formula that includes ginseng leaves, improved memory function in rats subjected to cerebral ischemia (Song et al., 2006). It is proposed that Jiannaoning acts by regulating the levels of proinflammatory cytokines—interleukin-2 (IL-2), IL-6 and neuropeptide Y in the rat brain. Moreover, ginsenosides inhibited the tumor necrosis factor-alpha (TNF-α) induced phosphorylation of IkappaB-α (IκB-α) kinase (IKK) and the subsequent phosphorylation and degradation of IκB-α. TNF-α-induced phosphorylation of mitogen-activated protein kinase kinase 4 (MKK4) and subsequent activation of the c-Jun N-terminal kinases activator-protein 1 (JNK-AP-1) pathway was also suppressed (Choi et al., 2007). Ginsenosides from ginseng leaves and stems upregulated the level of glucocorticoid receptor (GR) in the brain cytosol of heat-injured rats (Li et al., 2006). Interactions of ginsenoids or glucocorticoids with GR triggered cellular responses involving activation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway and increased nitric oxide (NO) production in human umbilical vein endothelial cells (Lee et al., 1997; Dancey, 2004). Ginsenosides may also regulate ion channels via their ligand-binding sites or channel pore sites (Nah et al., 2007). They inhibited voltage-dependent Ca^{2+}, K^{+}, and Na^{+} channel activities in a stereospecific manner (Liu et al., 2010) and blocked some subtypes of nicotinic acetylcholine (ACh) and 5-hydroxytryptamine type 3 receptors (Chen et al., 2010). Ginsenosides facilitated neurotransmission in the brain (Xue et al., 2006; Liu et al., 2010) but inhibited glutamate

TABLE 1 | Effect of ginsenosides on enzyme activities.

Enzyme	Effect	Reference
Superoxide dismutase	Stimulation	Fu and Ji (2003) and Sohn et al. (2013)
Glutathione peroxidase	Stimulation	Fu and Ji (2003) and Sohn et al. (2013)
Na^+, K^+-ATPase	Inhibition	Chen et al. (2009)
Phosphoinositol-3-kinase (Ptd Ins 3K)/Akt-dependent extracellular signal-regulated kinase 1/2	Stimulation	Kim et al. (2007)
Endothelial nitric oxide synthase	Stimulation	Kim et al. (2007)

excitotoxicity (Kim et al., 2002). In addition, ginsenosides have been reported to improve central cholinergic function, and are used to treat memory deficits in humans (Rudakewich et al., 2001). Ginsenosides increased levels of dopamine and norepinephrine in the cerebral cortex (Itoh et al., 1989) and have beneficial effects on attention, cognitive processing, sensorimotor function and auditory reaction time in healthy subjects (D'Angelo et al., 1986).

Bioavailability of Ginseng

The oral bioavailability of ginsenosides is very low. The poor bioavailability of ginsenosides is not only because of low membrane permeability and active biliary excretion, but also due to biotransformation (Liu et al., 2009). As mentioned above, deglycosylated products of ginsenosides are formed by their bacterial metabolism in the gut lumen. These products are more permeable and bioactive than glycosylated ginsenosides (Hasegawa and Uchiyama, 1998). Intestinal bacterial metabolites of ginseng are absorbed into the bloodstream. Quantitative and statistical analysis of ginsenosides in plasma indicates that protopanaxadiol (PD) saponins exhibit higher concentration and longer half-life than protopanaxatriol (PT) saponins (Zhang et al., 2014). The mean values of half-lives of the ginsenosides Rg1, Re, Rb1, Rb2/b3, Rc and Rd are 15.26, 2.46, 18.41, 27.70, 21.86 and 61.58 h; while their peak concentrations are 7.15, 2.83, 55.32, 30.22, 21.42, 8.81 μg/L respectively (Zhang et al., 2014). Studies on concentrations of ginsenosides in the brain at different time intervals after dosing show that ginsenosides enter into the brain rapidly, but concentrations decline rapidly with time. Ginsenosides with higher concentrations in the brain are Rg1, Re, Rb1 and Rc (Zhang et al., 2014). Rg1 and Re may be the main components directly affecting CNS neurons, due to their better brain distribution. On the other hand, ginsenosides of PD saponins may protect the brain mostly through a peripheral effect, due to their longer time in the circulation.

Molecular Mechanisms of Effects of Ginseng on the Brain

Effect on Glutamatergic Neurotransmission

Ginsenosides have a general *stimulatory* effect on the brain (Radad et al., 2011). Ginsenoside Rb1 increased glutamate release in neurons via the PKA-dependent signaling pathway, whereas ginsenoside Rg1 induced glutamate release in a calcium/calmodulin-dependent protein kinase II (CaMKII) dependent manner (Liu et al., 2010). Ocotillol is a derivative of pseudoginsenoside-F11, a ginsenoside found in American ginseng, and displayed excitatory effect on spontaneous action potential firing and depolarized the membrane potential of mitral cells. This effect was abolished by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the N-methyl-D-aspartate receptor (NMDAR) receptor antagonist D-AP5, suggesting a role for glutamatergic transmission in the effects of ocotillol (Wang et al., 2011b). Transmission of action potentials transmission in the somatosensory cortex was facilitated by ginsenoside Rb3 perfusion (Cui et al., 2012). Ginsenoside Rg1 supplementation improved the performance of aged mice in behavioral tests and significantly upregulated the expression of synaptic plasticity-associated proteins in the hippocampus, including synaptophysin, NMDAR subunit 1, postsynaptic density protein 95 (PSD95) and CaMKII alpha via the mammalian target of rapamycin (mTOR) pathway (Yang et al., 2014). Notoginsenoside R1, a major saponin isolated from *Panax notoginseng*, increased membrane excitability of CA1 pyramidal neurons in hippocampal slices by lowering the spike threshold, possibly through inhibition of voltage-gated K^+ currents. It also reversed $A\beta_{1-42}$ oligomer-induced impairments in long-term potentiation (Yan et al., 2014). Other studies showed that ginsenoside Rb1 inhibited the activity of L-type voltage gated calcium channels, without affecting N-type or P/Q-type Ca^{2+} channels in hippocampal neurons (Lin et al., 2012a). Gintonin is a newly identified compound from ginseng that is found to activate G protein-coupled lysophosphatidic acid (LPA1) receptors with high affinity (Hwang et al., 2012a; Nah, 2012). Gintonin stimulated neurotransmitter release (Hwang et al., 2015) and promoted synaptic enhancement via LPA1 receptor activation in central synapses (Park et al., 2015). Gintonin contains two components: "ginseng major latex-like protein 151" (GLP) and ginseng ribonuclease-like storage protein; and recent studies have determined the structure of ginseng major latex-like protein 151 and its proposed lysophosphatidic acid binding mechanism (Choi et al., 2015). The LPA1 receptor is localized in dendritic spines of cultured hippocampal neurons (Pilpel and Segal, 2006) that are sites of glutamatergic synapses, and further study is necessary to elucidate a possible role of gintonin and LPA receptor signaling, in the CNS effects of ginseng.

Effect on Monoamine Neurotransmission

Ginsenosides could have effects on monoamine signaling. Total saponins extracted from *Panax notoginseng* increased the levels of 5-hydroxytryptamine, dopamine and noradrenaline, suggesting that it may have antidepressant effects by modulation of brain monoamine levels (Xiang et al., 2011).

Effect on Estrogen Signaling

Improved object recognition and decreased immobility time in the forced swim test have been reported following ginsenoside Rb1 treatment. Pre-treatment with an estrogen receptor antagonist clomiphene blocked these effects, indicating that they are dependent on the estrogen receptor (Hao et al., 2011). Red ginseng also prevented downregulation of the ERβ estrogen receptor in immobilization-stressed mice (Kim et al., 2013b).

Effect on Nitric Oxide Production

Ginsenosides have been shown in many studies to *inhibit* inducible nitric oxide synthase (iNOS). Rg3 at 20 and 30 mg/kg oral doses modulated an increase in TNF-α, IL-1β and IL-6 mRNA after lipopolysaccharide (LPS) injection in mice (Park et al., 2012b). Protective effects of Re treatment occurred via the phospho-p38, iNOS, and cyclooxygenase-2 (COX-2) signaling pathways in LPS stimulated BV-2 microglial cells (Lee et al., 2012). A bacterial metabolite of Rg5 which is the main constituent of heat-processed ginseng, Rh3, also decreased the expression of iNOS and TNF-α and IL-6, and increased the expression of heme oxygenase −1(HO-1) in LPS stimulated BV-2 microglial cells (Lee et al., 2015). As with iNOS, neuronal nitric oxide synthase (nNOS) is *inhibited* by ginseng. Ginseng saponins are transformed by intestinal microflora, and a product Rh2 decreased the expression of nNOS in the hippocampus, while another product Rg3 decreased its expression in the neocortex (Jang et al., 2004). Ginsenoside Rg1 has neuroprotective effects on ischemia-reperfusion injury in cultured hippocampal cells by blocking calcium influx into neuronal cells and decreasing nNOS activity (He et al., 2014). In contrast to iNOS, and nNOS, ginsenosides appear to *stimulate* endothelial nitric oxide synthase (eNOS) and may have beneficial effects on the circulation. Ginsenoside-Rg1 increased the phosphorylation of GR, PI3K, Akt/PKB and eNOS, leading to NO production in human umbilical vein endothelial cells. Knockdown of GR modulated the Rg1 induced NO production. Results indicate that Rg1 can act as an agonist ligand for GR, and that activated GR can induce rapid NO production from eNOS via the PI3K/Akt pathway (Leung et al., 2006).

Effect on the Keap1/Nrf2 Adaptive Cellular Stress Pathway (Figure 2)

This is an important adaptive pathway in response to oxidative stress, and many studies have pointed to this pathway as a target of dietary phytochemicals (Reviewed in Lee et al., 2014). Under physiological conditions, Keap1 keeps the Nrf2 transcription factor in the cytoplasm, allowing it to be ubiquitinated and degraded by proteasomes; but when cells are exposed to neurodegenerative disease-mediated oxidative stress, a signal involving phosphorylation and/or redox modification in Keap1 blocks the enzymatic activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex, leading to decrease in Nrf2 ubiquitination and degradation. As a result, free Nrf2 translocates into the nucleus, where it transactivates the antioxidant response element (ARE) of many cytoprotective genes (**Figure 2**). Rg1 pretreatment induced Nrf2 nuclear translocation and activation of the PI3K/Akt pathway, which could antagonize iron-induced increase in reactive oxygen species (ROS) and decrease in mitochondrial transmembrane potential in cultured neurons (Du et al., 2013). Likewise, ginsenoside Rh3 increased Nrf2 DNA-binding activity and level of sirtuin 1 (SIRT1) resulting in inhibition of NF-κB in cultured cells (Lee et al., 2015). Protopanaxtriol extracted from *Panax ginseng* increased Nrf2 entering the nucleus, and induced the expression of phase II antioxidant enzymes, including HO-1 and nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidase 1, after 3-nitropropionic acid induced injury (Gao et al., 2015). Notoginsenoside R1 isolated from *Panax notoginseng* increased nuclear translocation of Nrf2 and ARE activity, and upregulated the expression and activity of HO-1, NADPH quinone oxidase 1 and gamma-glutamylcysteine synthetase (γ-GCSc) in PC12 cells (Meng et al., 2014). Together, results indicate an important role of the Keap1/Nrf2 pathway in neuroprotective effects of ginseng.

Effect on Neuronal Cell Survival

Oral administration of ginsenoside Rb1 significantly increased cell survival but not proliferation in the hippocampus, that may be related to its effects on learning and memory (Liu et al., 2011).

Effect on Apoptosis

Ginsenoside Rg induced downregulation of calpain I and caspase-3 and attenuated neuronal apoptosis caused by cerebral ischemia-reperfusion injury (He et al., 2012). Ginsenoside Rb1 is reported to suppress the activation of endoplasmic reticulum stress-associated proteins including protein kinase RNA (PKR)-like ER kinase (PERK) and C/EBP homology protein (CHOP) and downregulation of Bcl-2 induced by high glucose (Liu et al., 2013). *Panax notoginseng* saccharides increased Bcl-2/Bax ratio and had anti-apoptotic and neuroprotective effects after cerebral ischemia in rats (Jia et al., 2014).

Effect on Neural Stem Cells and Neuroregeneration

Ginsenoside Rb1 enhanced neurotrophin expression and induced differentiation of midbrain dopaminergic neurons (Hsieh and Chiang, 2014). In addition, Rg1 promoted neural stem cell differentiation through the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) and PI3K-AKT signaling pathways (Jiang et al., 2012a; Li et al., 2013). Ginsenoside Rd also increased neural stem cell proliferation (Lin et al., 2012b) and maintained neurogenesis after lead-induced neural injury (Wang et al., 2013b). *Panax notoginseng* saponins decreased the expression of the neurite inhibitory molecules Nogo-A and NgR after cerebral ischemia in rats, suggesting a role in axonal regeneration after injury (Liu et al., 2014).

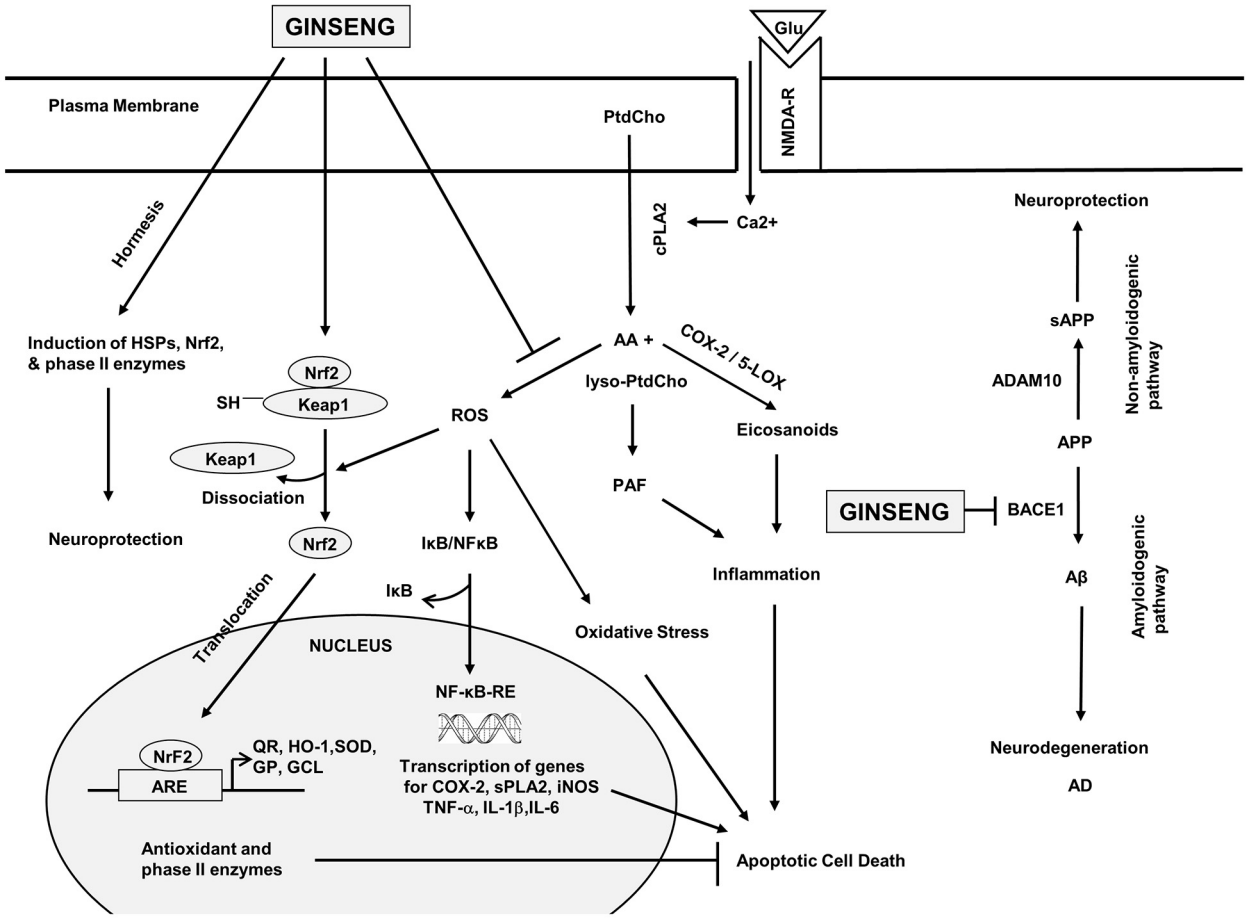

FIGURE 2 | Hypothetical diagram showing the effects of ginseng on signal transduction processes in the brain. phosphatidylcholine (PtdCho); lyso-phosphatidylcholine (lyso-PtdCho); cytosolic phospholipase A2 (cPLA2); arachidonic acid (AA); platelet activating factor (PAF); reactive oxygen species (ROS); Nuclear factor-kappa B (NF-κB); nuclear factor erythroid 2-related factor 2 (Nrf2); cyclooxygenase-2 (COX-2); lipoxygenase (LOX); secretory phospholipase A2 (sPLA2); inducible nitric oxide synthase (iNOS); tumor necrosis factor-α (TNF-α); interleukin-1β (IL-1β); interleukin-6 (IL-6); kelch-like erythroid Cap "n" Collar homologue-associated protein 1 (Keap1); NFE2-related factor 2 (Nrf2); antioxidant response element (ARE); quinine oxidoreductase (QR); hemeoxygenase 1 (HO-1); superoxide dismutase (SOD); glutathione peroxidase (GP); γ-glutamylcysteine ligase (γ-GCL); heat shock proteins (HSPs); amyloid precursor protein (APP); soluble amyloid precursor protein (sAPP); β-amyloid (Aβ); α secretase (ADAM10); and β-secretase (BACE1). This diagram is based on information provided in Choi et al. (2010), Jung et al. (2010), Li et al. (2010), Ye et al. (2011b), Hwang et al. (2012b), Karpagam et al. (2013) and Wang et al. (2013c).

Effect on Microglia

Microglia are resident innate immune cells in the CNS, which are activated in response to a variety of stimuli, such as infection, traumatic brain injury, toxic metabolites, or autoimmunity. Ginsenoside Rh1 inhibited the expression of iNOS, COX-2, and pro-inflammatory cytokines while increasing the expression of anti-inflammatory IL-10 in LPS-stimulated microglia. These effects were attenuated by inhibition of HO-1 (Jung et al., 2010). In addition, ginsenoside Rb1 reversed the changes in several direct or indirect neuroinflammatory markers in the hippocampus (Wang et al., 2011a). Ginsenoside Rb1 also decreased the expression of TNF-α, down regulated IL-6 expression, inhibited the activation of NF-κB and modulated microglial activation after brain ischemia (Zhu et al., 2012). Ginsenoside Rg1 pre-treatment of LPS induced BV-2 microglial cells activated the phospholipase C signaling pathway and modulated the expression of iNOS, COX-2, TNF-α, IL-1β and NF-κB (Zong et al., 2012). A ginseng saponin metabolite, compound K (20-O-D-glucopyranosyl-20 (S)-PD), reduced the number of Iba1-positive activated microglia, and inhibited the expression of TNF-α and IL-1β in the brain after LPS-induced sepsis (Park et al., 2012a). Together, results indicate that ginseng has anti-neuroinflammatory effects by reducing activation of microglia.

Effect on Astrocytes

Ginsenoside Rd increased levels of phosphorylated protein kinase B (PKB/Akt) and phospho-ERK1/2 (p-ERK1/2) and expression of glutamate transporter-1 (GLT-1) in astrocyte

TABLE 2 | Studies summarizing the effects of ginseng metabolites in neurological disorders.

Neurological disorder	Ginseng metabolite	Mechanism	Reference
Stroke	GinsenosideRd, GS-Rd, Ginsenoside Rb1	Anti-inflammatory; inhibition of Ca2+ influx, and reduction in edema	Ye et al. (2011a,b)
AD	Ginsenoside CK, F1, Rh1, and Rh2; *Panax notoginseng* saponins	Inhibition of Aβ aggregation, inflammation, and antioxidant effects	Karpagam et al. (2013) and Huang et al. (2014)
PD	Ginseng extract G115	Inhibition of α-synuclein aggregation	Van Kampen et al. (2014)
EAE and MS	Ginsenoside Rd	Regulation of IFN-γ and IL-4	Zhu et al. (2014)
Depression	Ginsenosides 20 (S) protopanaxadiol, Rg1, and Rb1	Antidepressant Upregulation of BDNF	Xu et al. (2010) and Wang et al. (2013c)

cultures after oxygen-glucose deprivation. The effect of Rd on GLT-1 expression was abolished by inhibition of PI3K/AKT or ERK1/2 signaling pathways (Zhang et al., 2013). Moreover, total saponins in the leaves of *Panax notoginseng* reduced H_2O_2 induced cell death in primary rat cortical astrocytes, likely through nuclear translocation of Nrf2 and upregulation of antioxidant systems including HO-1 and glutathione S-transferase (Zhou et al., 2014b). In contrast to normal or hypoxic astrocytes, ginsenoside Rg3 showed anti-cancer activities in malignant astrocytes glioblastoma multiforme cells, by induction of apoptosis via the methyl ethyl ketone (MEK) signaling pathway (Choi et al., 2013).

Effect on Oligodendrocytes and Myelination

Administration of American ginseng *Panax quinquefolium* to mice at a dose of 150 mg/kg body mass resulted in improvement of demyelination scores after induction of experimental autoimmune encephalomyelitis (EAE; Bowie et al., 2012).

Effect on Cerebral Microvessels

Ginsenosides may be effective in inhibiting changes to the cerebral microvasculature after cerebral ischemia. Weinaokang, a preparation consisting of active compounds extracted from *Ginkgo biloba*, ginseng, and *Crocus sativus* (saffron) inhibited the translocation of G protein-coupled receptor kinase 2 from the cytosol to the cell membrane, and reduced extracellular-signal-regulated kinases (ERK1/2) phosphorylation and matrix metalloproteinase expression in cerebral microvessels (Zheng et al., 2010). Ginsenoside Rg1 also modulated increase in aquaporin 4 expression and blood brain barrier (BBB) disruption after cerebral ischemia in rats (Zhou et al., 2014a).

Protective Effects of Ginseng on Neurological Disorders

Neurochemical Aspects of Neurodegenerative, Neurotraumatic, and Neuropsychiatric Disorders

Neurodegenerative diseases include AD, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. They are associated with progressive loss of cognitive function and motor disabilities with devastating consequences to the affected individual. Genetic factors, environmental factors and unhealthy lifestyle have been suggested to contribute to the pathogenesis of neurodegenerative processes (Farooqui, 2010; Seidl et al., 2014). Many neurodegenerative diseases are accompanied by oxidative stress and neuroinflammation, and associated with increased generation of lipid mediators, abnormal protein aggregation, slow excitotoxicity, loss of synapses and disintegration of neural networks, leading to failure of neurological functions (Jellinger, 2009; Farooqui, 2010). Neurotraumatic diseases are caused by metabolic or mechanical insult to the brain and spinal cord. They include cerebral ischemia or stroke, spinal cord injury and traumatic brain injury. Neurochemical events in neurotraumatic diseases include the release of glutamate, overstimulation of glutamate receptors, rapid calcium influx, activation of cytosolic phospholipase A_2 ($cPLA_2$), phospholipase C, COX-2 and NOS, induction of oxidative stress and neuroinflammation (Farooqui, 2010). Neuropsychiatric disorders include both neurodevelopmental disorders and behavioral or psychological difficulties such as depression, schizophrenia, and bipolar disorders. Impairment of cognitive processes results in behavioral symptoms such as abnormal thoughts or actions, delusions, and hallucinations. They involve abnormalities in the cerebral cortex, ventral striatum and other components of the limbic system (Gallagher, 2004).

Alzheimer's Disease (Table 2)

Cellular Changes Induced by Ginseng

AD is the most common form of dementia, a general term for memory loss and other intellectual abilities serious enough to interfere with daily life. It is characterized by the presence of extracellular plaques consisting of aggregated Aβ peptides and neurofibrillary tangles that contains hyperphosphorylated tau protein, as well as cerebral amyloid angiopathy, due to Aβ deposition around the walls of arterioles or capillaries in the brain (Reviewed in Cheng et al., 2005). Aβ peptides are generated from amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase activities, and are generally considered to be toxic to neurons, while neurofibrillary tangles interfere with cellular transport processes. Treatment

with *Panax notoginseng* flavonol glycosides inhibited the aggregation of Aβ in a dose-dependent manner and modulated the increase in Ca^{2+} and cell death triggered by Aβ in cultured neurons. These flavonol glycosides also reduced memory impairment in a passive avoidance task in scopolamine-treated rats (Choi et al., 2010). Treatment of SH-SY5Y neuroblastoma cells with gintonin, a novel LPA1 receptor activating ligand decreased Aβ1–42 release, and attenuated Aβ1–40 induced toxicity. In addition, long-term oral administration of gintonin attenuated amyloid plaque deposition, and short- and long-term memory impairment in a mouse model of AD (Hwang et al., 2012b). Fermented ginseng also reduced Aβ1–42 levels in HeLa cells stably expressing the Swedish mutant form of APP and decreased memory impairment in mouse models of AD (Kim et al., 2013a).

Effect on Aβ Formation

Ginseng may have beneficial effects in AD by reducing the formation of Aβ oligomers (**Figure 2**). Chronic supplementation with ginsenosides for 8 months in the drinking water modulated age-related memory impairment in mice (Zhao et al., 2011). *Panax notoginseng* saponins reduced brain APP mRNA levels and improved learning and memory in senescence-accelerated mice (Zhong et al., 2011), and daily consumption of American ginseng helped preserve cognitive functions in senescence-accelerated mice (Shi et al., 2012). Moreover, aged transgenic AD mice overexpressing APP/Aβ treated with ginsenoside Rg1 showed marked decrease in cerebral Aβ levels, reversal of neuropathological changes and protection of spatial learning abilities and memory (Fang et al., 2012). Ginsenosides CK, F1, Rh1 and Rh2 from *Panax ginseng* were found to have potential BACE1 inhibitory activities (Karpagam et al., 2013); and *Panax notoginseng* saponins increased non-amyloidogenic processing of APP by increasing α-secretase activity, and decreased amyloidogenic processing by decreasing BACE1 expression (Huang et al., 2014). Ginsenoside Rh2 improved learning and memory performance, and reduced the number of senile plaques in brains of Tg2576 mice (Qiu et al., 2014). Results suggest neuroprotective effects of ginsenosides by reducing Aβ levels via stimulation of α-secretase activity and inhibition of β-secretase activity.

Effect on tau Phosphorylation

Ginseng may also have a beneficial effect in AD by reducing tau hyperphosphorylation and neurofibrillary tangle formation. Total ginsenoside extracts from stems and leaves of *Panax ginseng* enhanced the phosphatase activity of purified calcineurin. This could be useful in AD, since inhibition of calcineurin leads to tau hyperphosphorylation at multiple sites in AD brains (Tu et al., 2009). Treatment with ginsenoside Rd cultured cortical neurons or AD rats (10 mg/kg for 7 days) reduced okadaic acid-induced neurotoxicity and tau hyperphosphorylation by enhancing the activities of protein phosphatase 2A (PP2A; Li et al., 2011a). Ginsenoside Rb1 reversed aluminum exposure-induced decreased PP2A level and tau phosphorylation in the cortex and hippocampus (Zhao et al., 2013). Ginsenoside Rg1 (20 mg/kg) reversed memory impairments induced by okadaic acid by decreasing levels of phosphorylated tau and suppressing the formation of Aβ in the brains of rats (Song et al., 2013). Results suggest neuroprotective effect of ginsenosides by reducing tau hyperphosphorylation.

Effect on Reactive Oxygen and Nitrogen Species

Ginsenosides are reported to have antioxidant effects. Rg1 is a potential regulator of hypoxia-inducible factor-1α (HIF-1α), and could act via this transcription factor to improve cell survival, angiogenesis and neurogenesis (Tang et al., 2011). Oral treatment with pseudoginsenoside-F11 (PF11), a component of *Panax quinquefolium* increased the activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and is associated with improved learning and memory in a mouse model of AD (Wang et al., 2013c). Ginsenoside Rg1 treatment modulated $Aβ_{25–35}$ induced mitochondrial dysfunction, inhibition of HIF-1α expression and increase in reactive nitrogen species and protein nitrotyrosination in human endothelial cells (Yan et al., 2013).

Effect on Cholinergic and Neurotrophic Signaling

Loss of ACh is found in the brain in AD. Ginsenoside Rg5 found in *Panax ginseng* modulated cognitive dysfunction and neuroinflammation in the brains of streptozotocin-induced memory impaired rats. Acetylcholinesterase (AChE) activity was reduced, while choline acetyltransferase (ChAT) activity was increased in the cortex of Rg5 treated rats (Chu et al., 2014). Ginsenosides Re and Rd also induced the expression of ChAT/VAChT and increased the level of ACh in Neuro-2a cells (Kim et al., 2014).

Clinical Studies on Use of Ginseng in AD

Patients in a high-dose Korean Red Ginseng group (9 g/day) showed significant improvement on the Alzheimer's Disease Assessment Scale (ADAS), Clinical Dementia Rating (CDR) after 12 weeks of Korean Red Ginseng therapy, when compared with those in the control group (Heo et al., 2008). In the long-term evaluation of the efficacy of Korean Red Ginseng in patients after 24 weeks, the Korean version of the Mini Mental Status Examination (K-MMSE) score remained without significant decline at the 48^{th} and 96^{th} weeks, and similar results were obtained with ADAS. Together, results point to long-term beneficial effects of Korean Red Ginseng in patients with AD (Heo et al., 2011).

Major Depression (Table 2)

Preclinical Studies

Major depressive disorder or major depression is a mental disorder characterized by a pervasive and persistent low mood that is accompanied by low self-esteem and by a loss of interest or pleasure in normally enjoyable activities. Ginseng total saponins at doses of 50 and 100 mg/kg for 7 days reduced the immobility time in the forced swim test, which is a mouse model of depression. They also reversed the reduction in the sucrose preference index, decrease in

locomotor activity, as well as prolongation of latency of feeding in a novel environment, in a chronic stress model of depression in rats (Dang et al., 2009). Ginseng saponins also had antidepressant effects in the corticosterone-induced mouse model of depression (Chen et al., 2014). Mice that received total saponins extracted from *Panax notoginseng* at doses of 10–1000 mg/kg daily for 1, 7, and 14 days showed decreased immobility time in the forced swim test. In the chronic mild stress model, chronic total saponin treatment (70 mg/kg) reversed depression-like behavior in rats. Levels of 5-hydroxytryptamine, dopamine, and noradrenaline were increased in the brains of treated rats, which could be the basis of the antidepressant effects (Xiang et al., 2011). Ginsenoside Rg1 upregulated BDNF signaling in the hippocampus, down regulated serum corticosterone levels, and reversed the decrease in dendritic spine density and hippocampal neurogenesis caused by chronic stress (Jiang et al., 2012b; Wang et al., 2013a). A post-metabolism compound and intestinal metabolite of ingested ginsenosides, PD, showed antidepressant effects in the tail suspension and forced swim tests, in the olfactory bulbectomy rat model of depression (Xu et al., 2010). Together with findings in the "Effect on Glutamatergic Neurotransmission" "Effect on monoamine Neurotransmission", "Effect on Estrogen Signalling", "Effect on Neural Stem Cells and Neuroregeneration" sections, they suggest that ginseng may have beneficial effects in major depression via multiple actions: (a) increasing AMPA receptor signaling, in a manner similar to AMPA receptor agonists or AMPAkines; (b) increasing the level of monoamines; (c) reducing stress related corticosteroid levels; and (d) increasing estrogen receptor signaling and BDNF, which are related to decreased neuronal death and improved neurogenesis.

Clinical Studies

A randomized, multicenter, double-blind parallel group study of 193 post-menopausal women treated with ginseng and 191 treated with placebo reported significant difference in depression scores in favor of ginseng compared with placebo (Wiklund et al., 1999). Clinical studies on 35 female outpatients aging from 18 to 65 years who were remitted from major depression with residual symptoms and given Korean Red Ginseng at a dose of 3 g/day reported significant decrease in depressive symptoms on the Depression Residual Symptom Scale (DRSS) and Montgomery-Åsberg Depression Rating over an 8-week period (Jeong et al., 2014). Another study of possible casual relation between hormones, energy sources, and minerals indicated that depression was related to serum lipids including cholesterol, and that fermented red ginseng had beneficial effects on depression by modulating this relation (Lee and Ji, 2014).

Stroke

Stroke or cerebrovascular event is associated with a sudden loss of brain function, due to disturbance in blood supply to the brain. Ginsenoside Rd (10–50 mg/kg) significantly decreased infarct volume and was associated with a better neurological outcome after transient middle cerebral artery occlusion (MCAO) in rats (Ye et al., 2011a). Interestingly, ginsenoside Rd demonstrated neuroprotection even when administered 4 h after recirculation of transient MCAO or after onset of ischemic stroke induced by permanent MCAO (Ye et al., 2011b). mRNA and protein expression levels of non-selective cation channels such as transient receptor potential melastatin (TRPM) and acid sensing ion channels (ASIC) were significantly increased 24 h following MCAO, and these effects were attenuated by treatment with 10 mg/kg ginsenoside Rd (Zhang et al., 2012). High dose ginsenoside Rb1 treatment reduced neurological deficits, brain edema, BBB disruption and the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells after hemorrhagic stroke (Li et al., 2010). Significant reduction in basilar artery vasospasm and lumen thickness was also observed after Rb treatment (Li et al., 2011b). Results suggest that neuroprotective effect of ginseng in stroke via inhibition of ion channels or modulation of vasospasm. It is, however, noted that ginseng may have a drug interaction with the anticoagulant warfarin that could lead to increased risk of bleeding, and this should be taken into consideration in patients who are on warfarin.

Parkinson's Disease

Parkinson disease is a neurodegenerative disorder affecting mainly the motor system, as a result of death of dopaminergic neurons in the substantia nigra. Ginsenosides investigated as neuroprotective agents for Parkinson's disease are Rb1, Rg1, Rd and Re. These compounds exert neuroprotective effects through inhibition of oxidative stress and neuroinflammation, decrease in toxin-induced apoptosis and nigral iron levels, and regulation of N-methyl-D-aspartate receptor channel activity (reviewed by González-Burgos et al., 2014). Oral administration of the ginseng extract, G115 modulated tyrosine hydroxylase-positive cell loss in the substantia nigra, and reduced locomotor dysfunction in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP^+) models of Parkinson's disease (Van Kampen et al., 2003). G115 also reduced dopaminergic cell loss, microgliosis, and accumulation of α-synuclein aggregates after chronic exposure to the dietary phytosterol glucoside, β-sitosterol β-d-glucoside or "BSSG model" of chronic Parkinson's disease (Van Kampen et al., 2014). MPTP administration resulted in behavioral impairment, dopaminergic neuronal death, and increased Cdk5 and p25 expression but decreased p35 expression in the nigrostriatal system of mice, and these effects were modulated by Korean Red Ginseng (Jun et al., 2015). Panaxatriol saponins, the main constituents extracted from *Panax notoginseng* provided neuroprotection against loss of dopaminergic neurons and behavioral impairment after MPTP treatment (Luo et al., 2011). Together, results suggest that ginseng may have neuroprotective effects in Parkinson's disease.

Multiple Sclerosis

Ginsenoside Rd has been studied in an animal model of multiple sclerosis, EAE. Intraperitoneally administered ginsenoside Rd at 40 mg/kg/day reduced the permeability of the BBB, regulated the secretion of interferon-gamma and IL-4, and decreased the severity of EAE in mice (Zhu et al., 2014).

Conclusions and Future Directions

Many ginsenosides have been isolated and characterized. The molecular mechanisms associated with ginsenosides involve scavenging free radicals, inhibition of inflammation, and prevention of excitotoxicity. Animal and cell culture studies have indicated that ginsenosides have different activities in both physiological and pathologic conditions. The structure activity relationship of ginsenosides has not been fully elucidated. However, it is becoming increasingly evident that ginsenosides produce neuroprotective effects by reducing free radical production and enhancing brain function. Studies involving each ginsenoside should include mechanisms of action, specificity, structure and function relationship, detailed pharmacokinetics and toxicity studies, and therapeutic studies in animal models and humans. Further studies are necessary to examine the effects of ginseng on metabotropic glutamate receptors and transporters, and the Keap1/Nrf2 adaptive cellular pathway. The neurological disorders for which there is most evidence from pre-clinical and a small number of clinical studies to benefit from ginseng are AD and major depression, and clinical trials are necessary to confirm the efficacy of ginseng and ginsenosides in the prevention and treatment of these, and possibly other neurological conditions.

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