INTERPHASE BETWEEN AGING AND NEURODEGENERATIVE DISEASES

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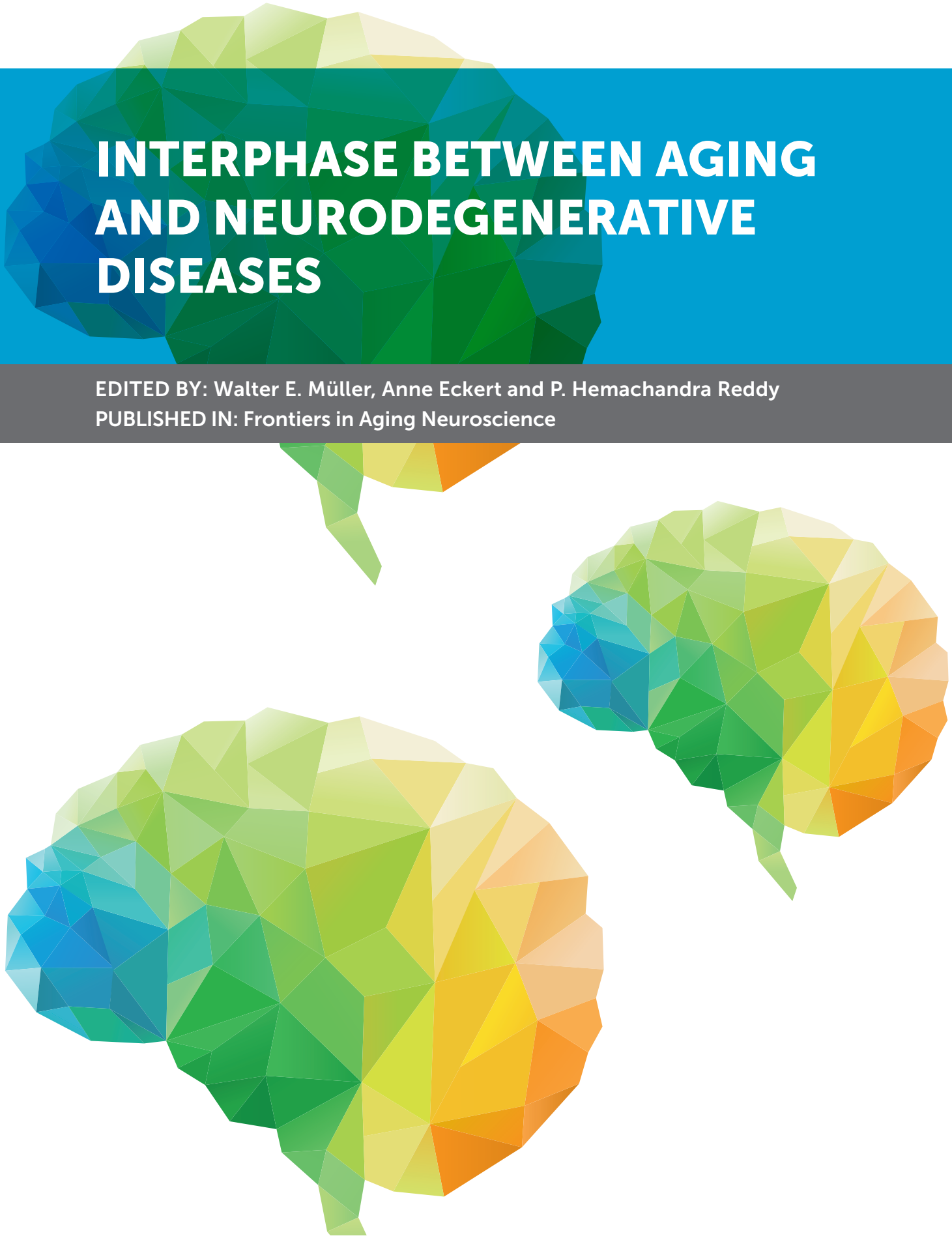

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INTERPHASE BETWEEN AGING AND NEURODEGENERATIVE DISEASES

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The purpose of this Research Topic is to discuss the latest developments in aging and neurodegenerative diseases. Aging represents the major risk factor of the two most relevant neurodegenerative diseases Parkinson's disease (PD) and Alzheimer's disease (AD). It is generally accepted that symptoms of PD correlate with the severity of degeneration of dopaminergic substantia nigra neurons. In most cases neuronal loss during aging is not sufficient to cause clinical symptoms but only leads to a preclinical state of PD. However, in a small number of our population, neurodegeneration by aging gets accelerated by individual (e.g. brain injuries), environmental (e.g. toxins) and genetic (e.g. mutations of the alpha-synuclein gene) factors to reach the critical threshold for clinical symptoms during lifetime. Thus, neurodegeneration in PD appears to represent the common final pathway of "normal brain aging" and all other risk factors including genetics and the accumulation of the neurotoxic alpha-synuclein protein.

While aging alone is generally agreed to be sufficient for at least the preclinical state of PD, the situation in AD seems to be different. Aging as the major and well documented risk factor of AD has been neglected for decades. Biochemical mechanisms of brain aging and the cognitive deficits of "normal brain aging" were seen as two not related and independent processes not related to AD. AD has always been characterized for decades by the presence of histopathological alterations (extracellular amyloid- containing plaques and intracellular tangles of hyperphosphorylated tau-protein), by neurodegeneration (synaptic deficits and finally neuronal loss), as well as by severe cognitive deficits clinically often accompanied by neuropsychiatric symptoms like delusions, as already described in the first famous patient Auguste D at the Psychiatric Hospital of Frankfurt.

If or if not one or both of the two histopathological hallmarks play a causative role remains unclear until now. The discovery of homocygotic risk genes in most of the very rare (probably less than 1%) cases of early onset AD which share increased production of β-amyloid (Aβ) as one (but probably not the only one) common property led to the hypothesis of Aβ as the major causative factor for the development of AD. It was neglected that plaques density in the brain of AD patients did not correlate with presence and severity of clinical symptoms, while synaptic deficits did so even in first observations already published many years ago.

Based on the Amyloid hypothesis, many drug treatments to remove Aβ plaques were developed. Even if all seemed to remove Aβ to some extent, all strategies

failed to improve the symptoms of dementia. Thus, other concepts to explain the development of clinical symptoms of AD over time are needed. These should include the brain aging process not only as a statistical but also as a causative contributing factor. These concepts should not only relay on cell or animal models but should much more take into account the disease and the patients. A closer look at the situation in PD will certainly be helpful.

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REVIEW
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Inflammation: Bridging Age, Menopause and APOEε4 Genotype to Alzheimer's Disease

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Neuro-inflammatory processes that contribute to development of Alzheimer's are evident early in the latent prodromal phase and worsen during the course of the disease. Despite substantial mechanistic and clinical evidence of inflammation, therapeutic approaches targeting inflammation have failed to alter the course of the disease. Disparate results from epidemiological and clinical trials targeting inflammation, highlight the complexity of the inflammatory process. Herein we review the dynamics of the inflammatory process across aging, midlife endocrine transitions, and the APOEε4 genotype and their contribution to progression of Alzheimer's disease (AD). We discuss the chronic inflammatory processes that are activated during midlife chronological and endocrine aging, which ultimately limit the clearance capacity of microglia and lead to immune senescence. Aging, menopause, and APOEε4 combine the three hits of a compromised bioenergetic system of menopause with the chronic low grade innate inflammation of aging with the APOEε4 dyslipidemia and adaptive immune response. The inflammatory immune response is the unifying factor that bridges across each of the risk factors for AD. Immune system regulators that are specific to stage of disease and inflammatory phenotype would provide a therapeutic strategy to disconnect the bridge that drives disease. Outcomes of this analysis provide plausible mechanisms underlying failed clinical trials of anti-inflammatory agents in Alzheimer's patients. Further, they highlight the need for stratifying AD clinical trial cohorts based on inflammatory phenotype. Combination therapies that include targeted use of anti-inflammatory agent's specific to the immune phenotype are considered.

Keywords: inflammation, Alzheimer's disease, menopause, APOEε4, aging

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INTRODUCTION

Alzheimer's disease (AD) is characterized by an extended prodromal phase of typically 10–20 years duration prior to clinical manifestation of cognitive decline (Amieva et al., 2008). The prodromal phase of AD consists of both- pre-stage symptoms and mild cognitive impairment (MCI) (Wilson et al., 2011). Clinical studies have shown that the prodromal phase is characterized by metabolic dysfunction, amyloid-β (Aβ) deposition in the brain, mild to moderate cognitive dysfunction, and chronic low-grade inflammation (Habeck et al., 2012; Olsson et al., 2013; Brinkmalm et al., 2014;

Wirz et al., 2014; Rajan et al., 2015; Mosconi et al., 2017a,b). These hallmark pathologies have aided in the development of biomarkers predictive of disease pathogenesis. In some populations, Aβ42 is one of the first biomarkers to appear in the cerebrospinal fluid (CSF) (Jack et al., 2013; Brinkmalm et al., 2014; Calsolaro and Edison, 2016). Despite the recent and widespread recognition of neuroinflammation in the pathogenesis of AD, there has been sparse expansion of inflammation-based biomarkers and preventive strategies. Understanding the dynamic interplay between inflammation and the risk factors such as age, APOE genotype, and endocrine transition states, can aid in this process. This review addresses the interaction between inflammation and risk factors key to the pathogenesis of AD. Strategies to specifically target these processes are also considered.

EARLY ACTIVATION OF INFLAMMATION AND RISK FOR ALZHEIMER'S DISEASE

Substantial evidence documents reactive microgliosis around plaque deposition and is now a hallmark of AD pathology (McGeer et al., 1988; Mattiace et al., 1990; Xiang et al., 2006). Reactive microgliosis and neuroinflammation in AD patients is considered a consequence of Aβ plaque deposition (McGeer et al., 1987). Microgliosis in AD is evidenced both microscopically and biochemically with increased levels of the proinflammatory cytokines including tumor necrosis factor-α (TNFα), IL-6, and IL-1β (Itagaki et al., 1989; Dickson et al., 1993; Ferretti and Cuello, 2011; Eikelenboom et al., 2012; Latta et al., 2014). While the inflammatory response to Aβ plaque deposition is irrefutable, it is a late stage response in the inflammatory cascade. Indicators of earlier inflammatory responses are apparent in multiple conditions that are risk factors for later development of AD.

Associations between the occurrence of systemic infections and chronic inflammatory conditions with Alzheimer's disease, suggests an active participation of inflammation in early stages of disease development. Patients with higher erythrocyte sedimentation rate (ESR), which is a clinical indicator of non-specific inflammation, are at greater risk of developing AD (Li et al., 2012). This is further corroborated by epidemiological studies that show that patients who suffer from chronic periodontal infection (Van Den Heuvel et al., 2007) and HIV have a higher risk of developing AD (Stanley et al., 1994; Alisky, 2007; Xu and Ikezu, 2009; Chakradhar, 2018). Recapitulating the clinical effect, Krstic et al. (2012) established an animal model that displayed AD like neuropathology by inducing chronic inflammation prenatally using a viral antigen, thereby showing that chronic inflammation potentiates the development of AD. Traumatic brain injury (TBI) also increases the risk of developing AD. Lesions that develop during TBI lead to an acute inflammatory response that includes microglial activation to facilitate debris removal and neuroprotection (Van Den Heuvel et al., 2007; Breunig et al., 2013; Habib et al., 2014). Incomplete resolution of the acute inflammatory response in TBI, however, is often followed by hypoxia and oxidative stress, which leads to the chronic activation of microglia and the release of neurotoxic proinflammatory cytokines (Van Den Heuvel et al., 2007; Breunig et al., 2013; Habib et al., 2014).

Chronic inflammatory conditions such as autoimmune disorders alter the risk of development of dementia. A recent study found patients admitted to the hospital for an autoimmune disorder have greater risk for subsequent hospitalization due to dementia (Wotton and Goldacre, 2017). This association was particularly significant for multiple sclerosis and systemic lupus erythematosus for AD. While patients with rheumatoid arthritis (RA) had a reduced risk of developing Alzheimer's disease, they had an increased risk of vascular dementia (Wotton and Goldacre, 2017). Multiple studies indicate that AD incidence is lower in persons with RA (Policicchio et al., 2017). Some attribute this reduction in incidence to the regular use of non-steroidal anti-inflammatory drugs (NSAIDs) (McGeer et al., 1996; Etminan et al., 2003). An alternative mechanism involves upregulation of granulocyte macrophage-colony stimulating factor (GM-CSF) with a probable gain of function in myeloid cells, thus enabling effective debris clearance is also hypothesized to reduce the incidence of AD in RA patients (McGeer et al., 1996; Boyd et al., 2010). In mice, increased levels of GM-CSF (both intrahippocampal and subcutaneous administration) significantly reduced amyloidosis and reversed cognitive impairment (McGeer et al., 1996; Boyd et al., 2010). More recent findings indicate that RA and risk of AD can be stratified based on treatment. Case-controlled study conducted on electronic medical records from 8.5 million commercially insured adults, indicate that RA patients treated with an anti-TNFα therapy, etanercept, had a lower risk of AD whereas those on other anti-inflammatory agents had increased risk of AD (Chou et al., 2016).

Disparate results from epidemiological studies and randomized clinical trials highlight the complexity of response to anti-inflammatory agents (Thal et al., 2005). Epidemiological analyses indicated that long-term NSAIDs users have a lower risk of developing AD (McGeer et al., 1996; Vlad et al., 2008). Based on epidemiological findings, a clinical study – ADAPT (Alzheimer's Disease Anti-Inflammatory Prevention Trial) was conducted in cognitively intact elderly individuals with a family history of AD. In this trial, the selective cyclooxygenase-2 (COX-2) inhibitor Celecoxib and non-selective COX inhibitor Naproxen, were used as preventive therapies. The trial was discontinued 15 months after randomization, due to the increased cardiovascular risk of these therapies. On extended follow-up after 7 years, treatment with celecoxib or naproxen for 1–3 years did not prevent cognitive decline (Group, 2007, 2008; Breitner et al., 2011; Alzheimer's Disease Anti-inflammatory Prevention Trial Research Group, 2013). Collectively, these findings indicate that timing of NSAID treatment for chronic inflammatory conditions are a critical factor impacting the efficacy of NSAID therapy to prevent or delay progression of Alzheimer's disease (McGeer et al., 1996; Vlad et al., 2008).

Discrepancies between the epidemiological and clinical trial findings indicate the need for greater refinement in considering patient populations and anti-inflammatory therapies. Elucidating the inflammatory phenotype which emerges during the

progression of AD requires consideration of the triggers that initiate chronic inflammation. In the sections below, we address how age, endocrine status, and APOE genotype impact inflammatory processes across AD progression from risk to late stage disease.

AGE-RELATED NEUROINFLAMMATION

Aging has a broad systems-level effect on human biology, which is evidenced by alterations in physiological function, metabolism, cognition, and inflammation (Smith et al., 2005). The effects of aging on immune responses are extensive and complex. In some individuals, adaptive immune responses will decline with age, whereas, others will experience aberrant immune responses leading to autoimmune disorders (Goronzy and Weyand, 2012; Vadasz et al., 2013; Fougère et al., 2017). Aging is associated with accumulation of oxidative stress and DNA damage and chronic low-grade inflammation (Cui et al., 2012). Though the effect of aging on cognitive function is variable, age remains the greatest risk factor for Alzheimer's in which inflammation is an early and persistent hallmark of the disease (von Bernhardi et al., 2015).

Central nervous system microglia plays a prominent role in innate immunity. Microglia constantly conducts surveillance of brain parenchyma to detect foreign pathogens and clear debris (Streit et al., 2004; von Bernhardi et al., 2015). Microglia detects and responds to a broad range of triggers including TBI, infections and damage associated molecular patterns (DAMPs). Reactive oxygen species (ROS), extracellular DNA and ATP all act as DAMPs (von Bernhardi et al., 2015; Gulke et al., 2018).

Innate immune responses by microglia are phenotypically typified by enlargement of the cell body, and molecularly by the upregulation of CD68, major histocompatibility complex-II (MHC-II) along with costimulatory molecules and secretion of pro and anti-inflammatory cytokines (Kim and Joh, 2006). The onset of innate immune responses leads to activation of the adaptive immune response. The innate activation of the adaptive immune response results in infiltration of peripheral immune cells, particularly T cell invasion of the brain (Kim and Joh, 2006). Together the innate and adaptive immune responses create the chronic low-grade inflammation typical of aging (Kim and Joh, 2006; see **Figures 1**, **2**).

Microglial phenotype is dynamic. It changes with age and is typified by both homeostatic and disease phenotypes (Butovsky et al., 2014; Keren-Shaul et al., 2017; Krasemann et al., 2017). The telomerase deficient accelerated aging mouse model exhibits reduced microglial numbers and deficient morphological and cellular processes (Khan et al., 2015). Further, microglial response to activation is stage of development dependent. Production of cytokines and trophic factors by microglia increases linearly with age (Lai et al., 2013). Microglia isolated from younger mice (2–4 months) exhibit a lower expression of proinflammatory cytokines: TNFα, IL-6, and IL-1β than older mice (Helenius et al., 1996; Crain et al., 2013; Latta et al., 2015). On activation by ATP, microglia isolated from neonatal rats and 13–15 month old adult rats have a more robust inflammatory response exhibited by upregulation of nitric oxide, TNFα, and brain derived neurotrophic factor (BDNF) in comparison to microglia derived from younger animals (2–11 months) (Lai et al., 2013). Aging distinctly affects migratory function of microglia; younger microglia on encountering activating signals exhibit an increase in motility and rapid extension of ramifications, whereas older microglia are less dynamic. Transcriptomic studies corroborate a reduction in migratory ability of microglia with age as age affects actin cytoskeleton reogranization which is vital in both phagocytosis and migration (Damani et al., 2011; Orre et al., 2014).

Comparison of the transcriptomic profiles of young and aged microglia revealed that microglial receptors (Trem2c, P2yr12, P2yr13, and Adora) involved in recognizing DAMPs such as, oxidized low-density lipoprotein, mitochondrial DNA, extracellular ATP decreased with age (Orre et al., 2014). In contrast, the expression of receptors that recognize pathogens and microbes (Tlr2, CD74, Ltf, Clec7a, Cxcl16, and Ifitm6) increases with age (Orre et al., 2014). Age-related changes in

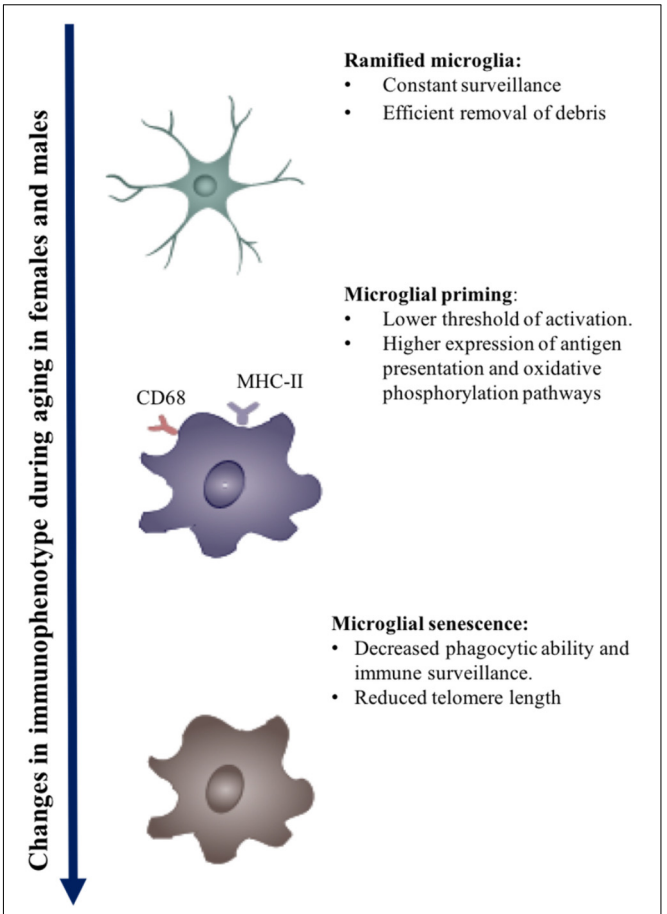

FIGURE 1 | Effect of aging on microglial immunophenotype. Changes in the immunophenotype of aging. Early to midlife aging can induced microglial priming, which is evident in the upregulation of MHC-II molecules, Fcg receptors, and microglial receptors. Chronic activation may eventually lead to immune senescence.

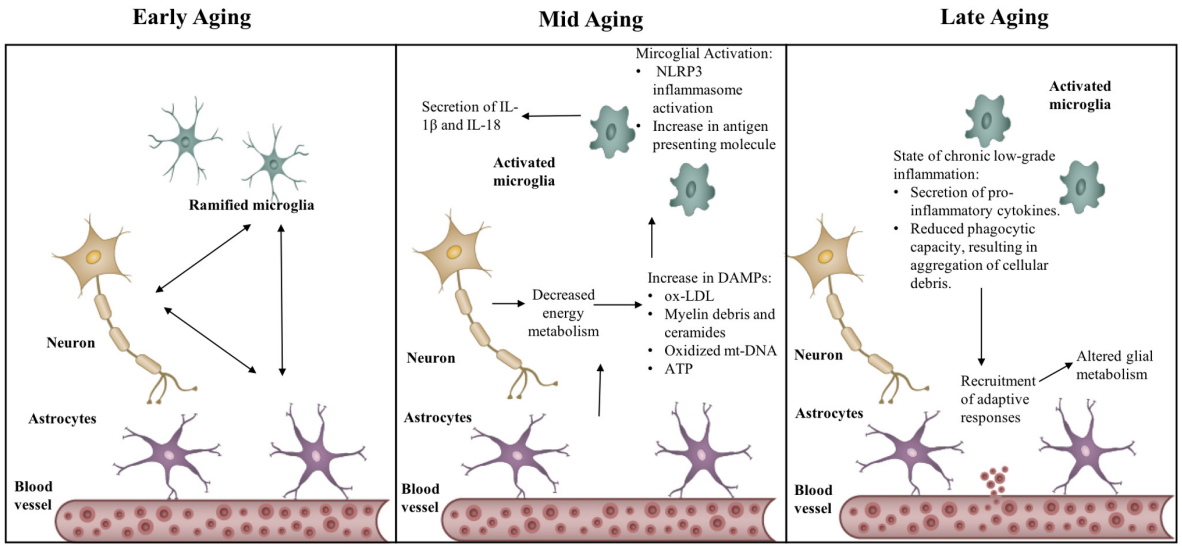

FIGURE 2 | Transitions in the inflammatory phenotype across aging. Aging causes activation of neuro-inflammatory mechanisms – NLRP3 inflammasome and increased MHC-II expression, through production of sterile triggers, such as, oxidized low-density lipoprotein (Ox-LDL), myelin debris, oxidized mitochondrial DNA (mt-DNA), ATP. State of chronic low-grade inflammation leads to recruitment of adaptive responses, in turn affecting glial metabolism.

microglial transcriptome are not ubiquitous as the expression of phagocytic receptors (Cd14, Cd68, Cd11b, and ICAM) remained unaltered in aging (Hickman et al., 2013; Smith and Dragunow, 2014). However, activation of microglial phagocytosis is diminished in aging. Studies characterizing microglial phagocytic capacity, report a reduction in phagocytosis with age which is especially evident following activation (Li M.D. et al., 2015; Ritzel et al., 2015). These findings indicate that despite stable expression of phagocytic receptors, the functional capacity of microglia decreases with age (Li M.D. et al., 2015; Ritzel et al., 2015). For example, the ability of microglia to phagocytose Aβ is affected by age, with microglia isolated from postnatal animals effectively phagocytosing Aβ fibrils, whereas adult microglia lose their capacity to do so (Floden and Combs, 2011). Other contributing factors to microglial senescence are age-related myelin degeneration and lysosomal storage in microglia, which in turn burden microglial clearance function (Holtman et al., 2015; Safaiyan et al., 2016).

Systemic inflammation and aging cause microglial priming. Primed microglia have a lower threshold for activation, are hypersensitive, develop an exaggerated immune response on activation, and have a distinct molecular signature from the M1–M2 phenotype (Perry and Teeling, 2013; Holtman et al., 2015; Ojo et al., 2015). The molecular signatures of primed microglia include the overexpression of antigen presentation, redox pathways, oxidative phosphorylation, and lysosomes.

In summary, during aging primed microglia generate a pro-inflammatory cascade due to their lower threshold of activation, enhanced reactivity, and limited functional capacity on encountering secondary triggers. The chronic activation of microglia coupled with age-related microglial priming hastens the process of senescence to cause loss of function over time (Franceschi et al., 2000; Streit and Xue, 2014). Senescent microglia appear to have lesser ramifications and stouter cell bodies, often referred to as dystrophic microglia.

Immunometabolic Sensor of Aging: Targeting Aging as a Disease

The inflammasome complex is a sensor of DAMPs. DAMPs act as an inflammatory challenge to the host defense mechanism and lead to the activation of the NLRP3 (Nod-like receptor pyrin domain 3) inflammasome complex (Youm et al., 2013; Zhang et al., 2013). Within the family of innate inflammasome sensors, the NLRP3 inflammasome has the unique ability to detect sterile inflammatory triggers. It can detect a wide range of metabolic and aging-related DAMPS, such as ROS production, glucose tolerance, and insulin resistance (Vandanmagsar et al., 2011; Salminen et al., 2012; Walsh et al., 2014), lipotoxic fatty acids, ceramides, free cholesterol, uric acid, and ATP, and it releases IL-1β and IL-18 (Youm et al., 2012, 2013; Zhang et al., 2013; see **Figure 2**).

The NLRP3 inflammasome complex activation is a two-step process. Molecular pathogens like lipopolysaccharides (LPS) have been shown to prime cells, leading to the activation of pattern recognition receptors (PRRs), the release of IL-1β, and increased expression of NLRP3. When followed by a secondary trigger such as ATP, this process causes the inflammasome to assemble and causes further activation. NLRP3 is also activated by the accumulation of damaged mitochondria due to the inhibition of autophagy, resulting in excessive production of ROS. oxidized mitochondrial DNA is also implicated in the activation of NLRP3 (Dixit, 2013). The activation of NLRP3 by ROS is mediated by thioredoxin interacting protein (TXNIIP) (Youm et al., 2012, 2013; Zhang et al., 2013).

The activation patterns of NLRP3 are similar in both macrophages and microglia. NLRP3 activation leads to the priming of microglia and reducing the threshold for activation (Halle et al., 2008; Heneka et al., 2013). Increase in caspase-1 activity in postmortem MCI and AD brains indicates the possible participation of the NLRP3 inflammasome in AD pathogenesis (Heneka et al., 2013). Targeting NLRP3 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) has been associated with a reduction in pathology of AD (Tang et al., 2015). In animal models carrying AD pathology, NLRP3 knockout and caspase-1 knockout caused spatial memory to remain intact. Moreover, the microglial phenotype in the NLRP3 knockout model shifted to the M2 anti-inflammatory phenotype with greater neuroprotection improved clearance of the plaque burden (Halle et al., 2008; Heneka et al., 2013).

With several studies linking NLRP3 inflammasome activation to chronic low-grade inflammation observed in aging, therapeutics targeting this sensor has also emerged. In a recent study, the ketone body – β-hydroxybutyrate (BHB) was found to suppress NLRP3 activation caused by urate crystals, lipotoxic fatty acids, and ATP. The levels of BHB increase with starvation, caloric restriction, and high-intensity exercise. Aging also marks a shift in the fuel usage and dependence on different fuel mechanisms. The inhibition of NLRP3 by BHB resulted in a decrease of IL-1β and IL-18 production by monocytes. BHB also reduced caspase-1 activation and IL-1β secretion in mouse models of NLRP3-mediated chronic inflammatory diseases like Muckle-Wells syndrome (Youm et al., 2015).

Another molecule, MCC950, blocked canonical and non-canonical activation of NLRP3 and attenuated experimental autoimmune encephalomyelitis (EAE). Both, MCC950 and BHB, were used in a mouse model of Muckle-Wells syndrome, which is characterized by chronic inflammation mediated by NLRP3. Thus, targeting NLRP3 in aging and aging-related disorders could be an important therapeutic strategy (Coll et al., 2015).

MENOPAUSE, INFLAMMATION, AND AD

The endocrine transition of the perimenopause to the post-menopause, while associated with loss of reproductive function (Brinton et al., 2015), is also associated with rise in chronic low-grade inflammation (Yin et al., 2015). Chronic systemic inflammation accelerates ovarian failure (Ağaçayak et al., 2016). Conversely, depleting proinflammatory cytokines IL-1α and IL-1β extends ovarian function and lifespan (Uri-Belapolsky et al., 2014). Concurrent with the chronic low-grade inflammation, the perimenopausal transition is typified by decline in brain glucose metabolism and mitochondrial respiration (Yao et al., 2010; Ding et al., 2013; Brinton et al., 2015; Yin et al., 2015; Mosconi et al., 2017a,b), myelin catabolism (Klosinski et al., 2015) and loss of white matter volume (Mosconi et al., 2017a,b), beta-amyloid deposition in brain (Mosconi et al., 2017a,b) and changes in neurological function (Brinton et al., 2015). Later age at natural and surgical menopause is associated with better verbal memory (Kuh et al., 2018). Surgically induced menopause prior to natural menopause is associated with rapid cognitive decline and earlier onset of AD (Rocca et al., 2008; Bove et al., 2014). Further, studies have shown that post-menopausal women with higher estradiol levels have a reduced risk of developing AD (Manly et al., 2000).

Post-menopausal women are at higher risk for developing autoimmune disorders and obesity (Doran et al., 2002; Bove, 2013). The incidence of RA is higher in peri- and post-menopausal women (Doran et al., 2002). The pathology of multiple sclerosis worsens after menopause (Tutuncu et al., 2013). Post-menopausal women are more prone to robust immune responses. The lack of ovarian steroidal hormones potentiates the inflammatory process predisposing menopausal women to immune disorders (Benedusi et al., 2012; Kireev et al., 2014; Sharma et al., 2018). Menopause and the associated lack of steroidal hormones further potentiate inflammation, which is reflected in levels of circulating cytokine levels and inflammatory responses. IL-6 and sIL-6 levels are higher in postmenopausal women (Giuliani et al., 2001). IL-4 and IL-2 levels also increase with menopause, which can be reversed by hormone therapy (Yasui et al., 2007). Serum IFN-γ levels increase during early menopause but decrease in later menopause (Goetzl et al., 2010). Peripheral blood mononuclear cells (PBMCs) isolated from postmenopausal women produced higher IL-6, IL-1β, and TNFα upon induction by LPS than PBMCs isolated from premenopausal women (Brooks-Asplund et al., 2002).

In addition to an altered cytokine profile, changes in T cell biology occur in women during this endocrine transition. Pre-menopausal women have higher CD4 counts than men and thus a more robust response to vaccination. Menopause causes a reduction in CD4 T cell numbers. This eventually causes an inversion of the CD4/CD8 T cell ratio, which is indicative of aging and can be correlated with increased oxidative stress (Larbi et al., 2008; Gameiro et al., 2010; Muller et al., 2015). The number of B2 cells (involved with antibody production) also decreases with menopause, especially during late menopause in comparison to perimenopause (Kamada et al., 2001). In mice, ovariectomy causes a reduction in the LPS-induced proliferation of leukocytes and subsequent chemotaxis, which is indicative of premature immune senescence (Baeza et al., 2011). Ovariectomized animals generally have a reduced and delayed adaptive response to vaccination, leading to decreased IgG titers in comparison to animals with intact ovaries (Haberthur et al., 2010). These changes are indicative of immune senescence occurring during menopausal transition. In the context of AD, the systemic effect of menopause on inflammation combined with effects on neurological function indicates cruciality of the menopausal transition in AD pathogensis.

Molecular Neuro-Inflammatory Mechanisms in Menopause

Menopause is composed of three transitions; the perimenopause that precedes menopause, the cessation of reproductive capacity, menopause, and the years following menopause, post-menopause. Concomitant with this endocrine aging is chronological aging as the endocrine transition states span

multiple years. Each of these endocrine stages is characterized by complex hormonal fluctuations (Brinton et al., 2015).

Decline in estradiol level during perimenopause and menopause coincides with a bioenergetic deficit in brain (Ding et al., 2013). Estradiol is a master regulator of metabolic function in the female (Rettberg et al., 2014). Clinical evidence of decline in glucose metabolism in brain and the coincident bioenergetic deficit is evidenced by reduced uptake of 18-fludeoxyglucose detected by positron emission tomography (PET) in perimenopausal and menopausal women (Mosconi et al., 2017a,b). The bioenergetic deficit precedes a shift to utilization of ketone bodies as a compensatory response to decline in brain glucose as bioenergetic fuel to generate ATP in brain. This shift to utilizing an auxiliary fuel during female endocrine aging activates catabolism of white matter as an endogenous lipid source of ketone bodies in brain (Klosinski et al., 2015) and a concomitant increase in microglial and astrocytic reactivity (Xie et al., 2013; Suenaga et al., 2015).

Analyses of microarray data obtained from the brains of postmenopausal women made available by NCBI revealed an inflammatory gene expression profile in the post central and superior frontal gyrus (Sárvári et al., 2012). In comparison to pre-menopausal women, post-menopausal women showed an upregulation in microglial markers CD14, CD18, and CD45, as well as TLR4 and MHC-II markers CD74 and C3 (Sárvári et al., 2012). These findings in the human female brain were consistent with the pattern of gene expression in the frontal cortex of ovariectomized middle-aged rats (13 months old) (Sárvári et al., 2012). Ovariectomy caused an upregulation of microglial reactivity markers CD11b, C18, CD45, and CD86, complement pathway C3, and phagocytic markers Msr2 and CD32. Together, these data are indicative of a shift in the microglial phenotype to an activated state (see **Figure 3**).

Hippocampal inflammatory gene expression in middle-aged rats drastically changed upon ovariectomy. A similar upregulation of microglial markers (CD45, IBA1, CD68, CD11b, CD18, Fcgr1a, and Fcgr2b) to that witnessed in the cortex was observed in the hippocampus. The gene expression results imply a possible activation of microglia. This effect was mitigated by treatment with estradiol and selective estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) agonists (Sarvari et al., 2015). Human post-menopausal gene expression in the hippocampus corroborated the inflammatory gene expression pattern in ovariectomized rats, with an upregulation of microglial reactivity markers CD11b, CD18, IBA1, CD14, and complement C3 (Sarvari et al., 2015).

In parallel, aging is associated with a marked upregulation in genes encoding the major histocompatibility complex class I and class II (MHC-I and MHC-II) (VanGuilder et al., 2011), alterations in Toll-like receptors (TLRs) (Shaw et al., 2011), and the complement pathway (Reichwald et al., 2009). This effect is more pronounced in women and represents the sexual dimorphism of the immune system (Blalock et al., 2003; Berchtold et al., 2008). The dynamics involved between age and menopause-related increase in myelin degeneration and microglial priming can be a tipping point in the neuro-inflammatory system. The increased myelin antigen load and upregulation of antigen presentation by microglia can set

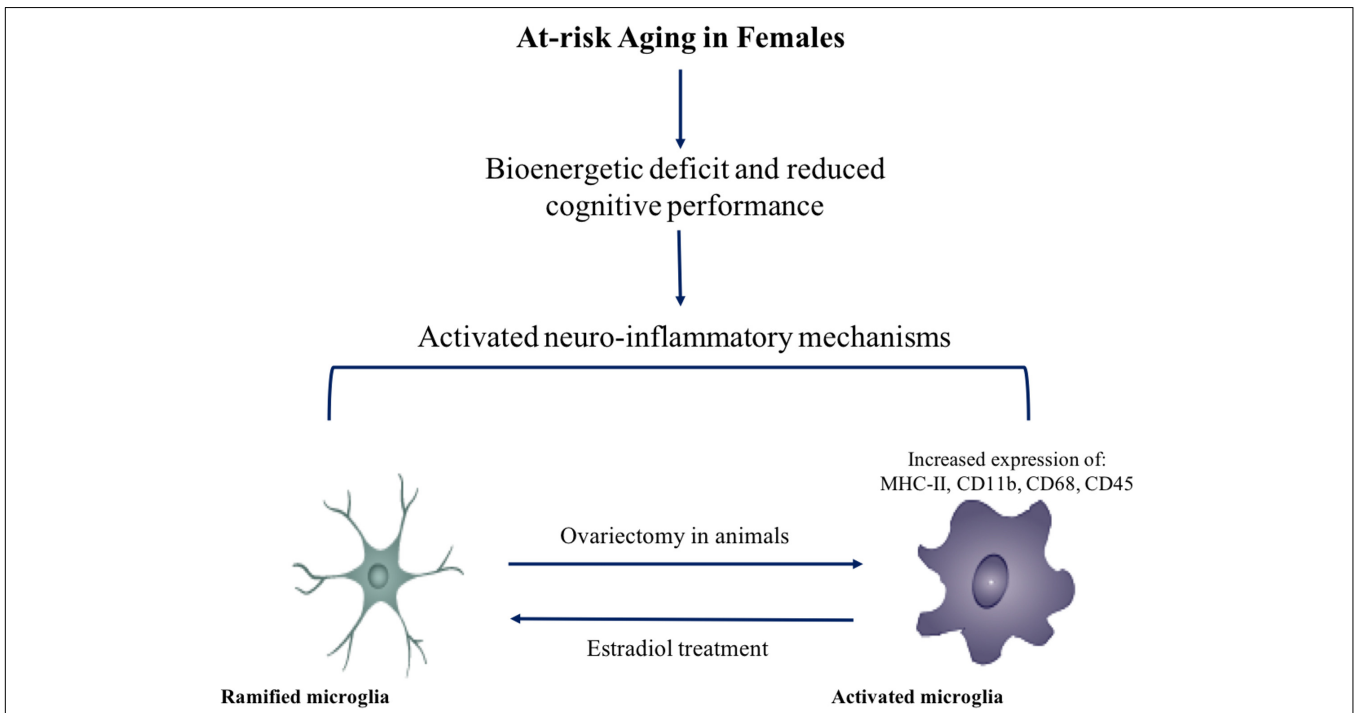

FIGURE 3 | Effect of menopause on immunophenotype. Decline in ovarian hormones induces a pro-inflammatory state in microglia. Activation of microglia and neuro-inflammatory mechanisms may be involved in at-risk aging females.

forth a cascade that leads to dysregulated glial metabolism and hypertrophy, eventually causing altered extracellular matrix (Blalock et al., 2003). Each event is pivotal in the development of AD.

Hormone Therapy: A Potential Anti-inflammatory Preventive Intervention for Alzheimer's Disease?

Hormone therapy (HT) promotes neuronal survival and has been shown to improve cognitive function and episodic memory in perimenopausal and postmenopausal women (Morrison et al., 2006; Brinton, 2008). Epidemiological studies have shown that HT delays the onset of AD as well as reduces the risk of developing AD (Tang et al., 1996; Persad et al., 2009; Dye et al., 2012). Women transitioning through their menopause benefit most from HT as compared to women who have already transitioned (Girard et al., 2017). Results from several clinical trials have emphasized on the timing of treatment with HT and the drawbacks of missing the window of treatment (Hogervorst et al., 2000; Zandi et al., 2002; Shumaker et al., 2003). This effect of estradiol in HT has been explained by two theories: the healthy cell bias of estrogen action (Brinton, 2008) and critical window hypothesis (Maki, 2013). Healthy cell bias highlights that neuronal viability and health at baseline are important for estradiol to exert its therapeutic efficacy, whereas the critical window hypothesis focuses on the perimenopausal transition, when cells are still healthy, being a key phase for using HT. The use of estradiol in HT provides a therapeutic opportunity to target inflammatory pathways that simultaneously modulate metabolic functions, thereby providing a supportive milieu for neuronal survival and growth (Vegeto et al., 2008; Zhao et al., 2014). HT restores the hormonal levels in post-menopausal women to those of premenopausal women. Post-menopausal women using HT have higher lymphocyte numbers and higher circulating monocytes in comparison to post-menopausal women who are not on HT (Kamada et al., 2000). Likewise, levels of B2 cells involved in antibody production are significantly higher in HT users in comparison to non-users (Kamada et al., 2001).

Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are abundantly expressed in astrocytes, microglia, and neurons, and both ERα and ERβ are involved in regulating the immunomodulatory responses exerted by astrocytes and microglia (Liu et al., 2003; Khan and Ansar Ahmed, 2015). In ovariectomized middle-aged rats, estradiol induces downregulation of the complement pathway and macrophage-associated genes in the frontal cortex. This effect was mediated through ERα and ERβ (Sárvári et al., 2011). Estradiol treatment in microglial cells induces a dose-dependent attenuation in superoxide release, phagocytic activity, and a concomitant increase in iNOS activity, without altering NF-κB expression (Bruce-Keller et al., 2000; Drew and Chavis, 2000). Some studies have shown that sex steroids reduce neuroinflammation via inhibiting the inflammasome complex, a possible downstream effect mediated by ERα and ERβ (Slowik and Beyer, 2015).

Astrocytes also participate in mediating the neuroprotective anti-inflammatory effect of estradiol via ERα (Spence et al., 2011). In contrast to microglial cells, estradiol inhibits NF-κB expression in astrocytes (Giraud et al., 2010; Acaz-Fonseca et al., 2014). Estradiol inhibits secretion of proinflammatory cytokines IL-6, TNF-α, IL-1β, expression of matrix metalloproteinases 9 (MMP-9), and interferon gamma-inducible protein 10 (IP-10) in astrocytes. Estradiol also reduces proinflammatory cytokines secreted by astrocytes when exposed to Aβ (Giraud et al., 2010; Acaz-Fonseca et al., 2014).

Much like estradiol, selective estrogen receptor modulators (SERMs) exert a neuroprotective effect by reducing neuroinflammation. Tamoxifen and raloxifene both reduce microgliosis, astrogliosis, and the production of proinflammatory cytokines IL-6 and IP-10 induced by LPS administration (Arevalo et al., 2012). They have also been demonstrated to protect neurons against neurotoxicity caused by neuroinflammation through an ER mediated pathway (Ishihara et al., 2015). SERMs reduce the proinflammatory response produced by astrocytes and are helpful in potentiating their neurotrophic function (Tapia-Gonzalez et al., 2008; Cerciat et al., 2010; Arevalo et al., 2012; Ishihara et al., 2015).

APOEε4, INFLAMMATION, AND ALZHEIMER'S DISEASE

APOEε4 is the primary genetic risk factor for the late onset form of AD (Scacchi et al., 1995; Liu et al., 2014; Manning et al., 2014). The human form of the APOE gene possesses three polymorphic alleles: E2, E3, and E4. The E3 allele occurs more frequently (77%) than E2 (8%) and E4 (14%) (Eisenberg et al., 2010). The E4 allele occurs in 40% of AD patients (Farrer et al., 1997). However, 91% of homozygous E4 carriers and 47% heterozygous carriers go on to develop AD (Corder et al., 1993).

Female APOEε4 carriers are more susceptible to developing AD than males (Altmann et al., 2014; Ungar et al., 2014; Neu et al., 2017). Prospective cohort studies have also suggested that female APOEε4 carriers are at greater risk of converting from MCI to AD than males (Altmann et al., 2014). Female APOEε4 carriers also have a higher rate of cognitive decline than APOEε3 carriers (Holland et al., 2013). Leukocyte telomere length is also greatly reduced in APOEε4 carrier's relative to age-matched controls, reflecting premature aging of female APOEε4 carriers (Jacobs et al., 2013).

Apolipoprotein E (ApoE) is a key regulator of lipid homeostasis and in the brain, it functions to shuttle lipid molecules from astrocytes and microglia to neurons, via lipoprotein complexes (Liu et al., 2013). In the periphery, ApoE is expressed in macrophages and liver. In the central nervous system, ApoE is mainly produced by astrocytes and microglia (Liu et al., 2013).

Sources of Neuroinflammation in APOEε4

ApoE is known to exert an immunosuppressive effect by inhibiting lymphocyte proliferation, Ig synthesis, and neutrophil activation. ApoE also exerts this immunosuppressive property on microglial activation (Guo et al., 2004; Baitsch et al., 2011;

Christensen et al., 2011). Relative to APOEε2 and APOEε3 carriers, APOEε4 carriers generate less ApoE protein in periphery and brain (Larson et al., 2000). Given the reduced amounts of ApoE protein in APOEε4 carriers, this population could be predisposed to a heightened inflammatory response when encountering a sterile inducer or infection (Ukkola et al., 2009; Gale et al., 2014; Tai et al., 2015).

Due to the differences in cysteine and arginine residues translated at positions 112 and 158, each of the three different isoforms of APOE exhibit different conformations. The conformational change in the protein affects its stability, folding characteristics, and the propensity to bind lipoprotein particles (Jofre-Monseny et al., 2008). APOEε4 has a globule-like structure and preferably binds to very low-density lipoprotein (vLDL) and low-density lipoprotein (LDL) particles. However, APOEε2 and APOEε3 tend to bind high-density lipoprotein (HDL) particles (Jofre-Monseny et al., 2008). This difference in protein structure affects the lipid shuttling ability of ApoE, which leads to hypercholesterolemia in APOEε4 carriers and increases the predisposition for generation of plaques (Jofre-Monseny et al., 2008). ApoE also exerts an inhibitory effect on the oxidation of LDL in an isoform-specific manner (E2 > E3 > E4) (Miyata and Smith, 1996). Among smokers, APOEε4 carriers have significantly higher amounts of oxidized LDL (ox-LDL) (Jofre-Monseny et al., 2008).

Cholesterol, ox-LDL, and Aβ are sterile inducers of inflammation called DAMPs (Miller et al., 2011; Clark and Vissel, 2015). DAMPs are recognized by PRRs expressed on macrophages, dendritic cells, monocytes, microglia, and neutrophils, which trigger the activation of an inflammatory process (Silverstein and Febbraio, 2009). One such PRR is CD36. In the context of recognizing DAMPs and plaque formation, it was recently demonstrated that CD36 (expressed on monocytes, macrophages, and microglia) recognizes soluble ligands such as oxidized LDL and soluble Aβ and converts them to crystals and fibrils, respectively. This leads to the assembly and activation of the NLRP3 inflammasome and the consequent release of the proinflammatory cytokine IL-1β (Sheedy et al., 2013; Oury, 2014).

Due to the increased probability of APOEε4 carriers to develop plaques, cholesterol crystals, and amyloid depositions, cellular immune function and reactivity are affected. In APOEε4 carriers there is a reduced clearance and efflux of cholesterol from macrophages (Cash et al., 2012). Moreover, increased nitric oxide production in APOEε4 causes increased platelet aggregation and secretion of adhesion molecules, further enabling the plaque formation in the periphery. In the brain, microglial and astrocytic clearance of debris is also diminished (Guo et al., 2006).

ApoE also affects Aβ uptake and oligomerization and thus can be a key factor in Aβ turnover. AD patients possessing the APOEε4 allele were found to have higher levels oligomeric Aβ in their brains as compared to APOEε3 carriers, implicating an association between ApoE with Aβ (Hashimoto et al., 2012). *In vitro* experiments correspond to clinical findings and have shown that APOEε4 has the greatest effect on promoting Aβ oligomerization in comparison to other isoforms (Hashimoto et al., 2012). Blocking Aβ and ApoE interaction by ApoE Aβ antagonist in hippocampal neuronal and astrocytic co-culture systems led to decreased accumulation and oligomerization of Aβ. Treatment with ApoE Aβ antagonists also inhibited the loss of synaptic proteins induced by Aβ accumulation (Kuszczyk et al., 2013; Liu S. et al., 2014).

Coupled with the Aβ oligomerization, ApoE also affects Aβ uptake. Astrocytes secrete ApoE as a lipoparticle into the interstitial fluid, where it binds with Aβ. Neurons endocytose and internalize these lipoparticles, thus promoting Aβ uptake. APOEε4 isoform has maximal binding affinity to Aβ and thus causes a greater uptake of Aβ by neurons in comparison to other isoforms (Mulder et al., 2014). APOEε4 prevents the uptake of oligomeric Aβ by astrocytes and the uptake of both oligomeric and fibrillar Aβ by microglia thereby inhibiting its clearance (Mulder et al., 2014).

ApoE also modulates Aβ clearance by microglia by regulating Aβ clearing enzymes such as neprilysin intracellularly and insulin degrading enzyme extracellularly. Effective degradation of Aβ depends on Liver X Receptor (LXR) activation, the isoform of APOE expressed, and the lipidation status of ApoE particles (Hashimoto et al., 2012; Kuszczyk et al., 2013; Liu S. et al., 2014). Activation of LXR/RXR (Retinoid X receptor) potentiates Aβ clearance as it compensates for the loss of APOEε4 function and induces the expression of ATP-binding cassette transporter subfamily A member 1 (ABCA1) and ApoE, thus inducing clearance by microglia and astrocytes (Lefterov et al., 2007; Terwel et al., 2011; Lee et al., 2012; Mandrekar-Colucci et al., 2012; Tai et al., 2014). Therefore, the presence of APOEε4 promotes the production of Aβ and uptake by neurons while preventing clearance and enabling the production of DAMPs and chronic low-grade inflammation.

Given the dysregulated lipid metabolism and impairment of cellular function with age, the APOEε4 allele is associated with increased systemic inflammation. It is expected that this would be reflected in cytokine levels, which are inflammatory markers such as C-Reactive Protein (CRP) measured in plasma/serum. Surprisingly, however, this is not the case, at least with CRP. Studies have consistently shown that CRP levels are lower in APOEε4 carriers (Ukkola et al., 2009; Lima et al., 2014; Metti et al., 2014; Yun et al., 2015). Marz et al. (2004) proposed that the metabolism of CRP might be associated with the mevalonate/cholesterol synthetic pathway, which might be downregulated in APOEε4 carriers. On the other hand, studies have shown that levels of IL-1β and vascular inflammatory marker: vascular cell adhesion molecule-1 (VCAM-1) are higher in E4 carriers (Olgiati et al., 2010; see **Figure 4**).

The APOEε4 allele also accelerates aging, which is reflected in the shorter telomeres in APOEε4 women in comparison to APOEε3 women (Jacobs et al., 2013). The accelerated aging phenotype is also evident in the reduction of T cell numbers. Age-related reduction in T cells for women is more dramatic during menopause, which is even more pronounced if the woman is an APOEε4 carrier (Begum et al., 2014). In comparison to APOEε3 derived microglia, estradiol has a reduced anti-inflammatory effect on microglia derived from APOEε4 (Brown et al., 2008). The APOEε4 allele is also a risk factor for metabolic syndrome and has been associated with RA, thus

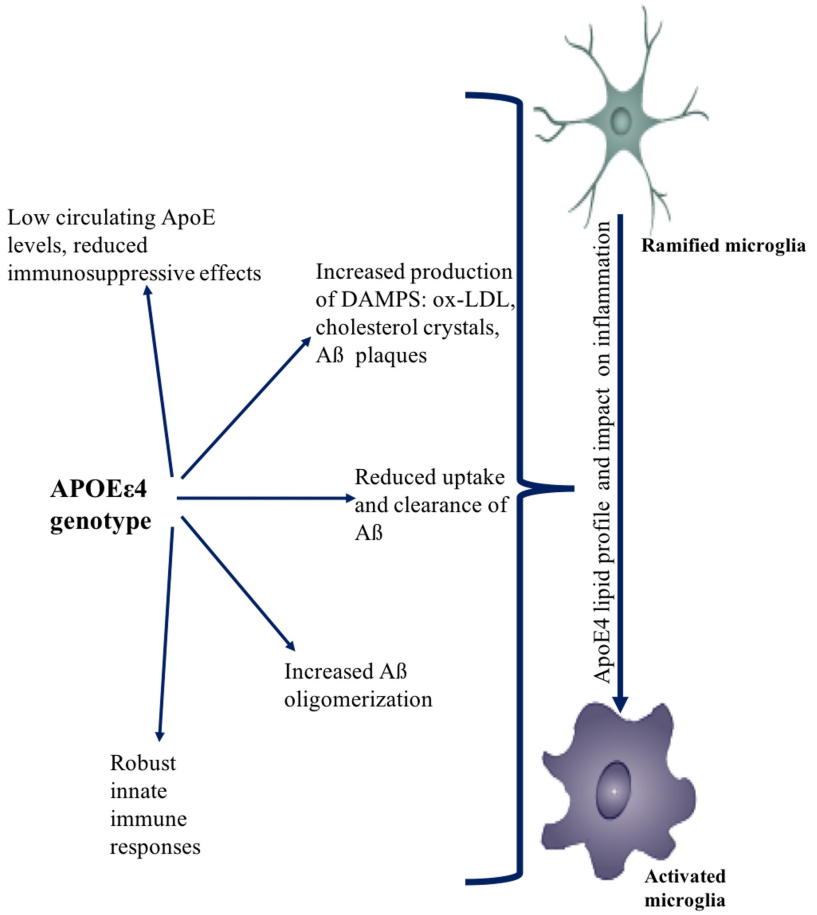

FIGURE 4 | Effect of APOEε4 on immune function. APOEε4 genotype affects the uptake, clearance, and production of sterile triggers of inflammation. APOEε4 causes increased accumulation of DAMPs such as Aβ, ox-LDL, cholesterol. APOEε4 also causes more robust innate immune responses. Collectively, the effects of APOEε4 genotype promotes an overall neuro-inflammatory state.

increasing the risk of comorbidities that can, in turn, affect systemic inflammation (Sima et al., 2007; Gungor et al., 2012; Toms et al., 2012; Johnson et al., 2013). APOEε4 also affects immune function by causing blood barrier dysfunction (Nelson et al., 2016).

Innate Immune Responses in APOEε4

Inflammatory responses triggered by innate immune agonists are highest in APOEε4 carriers (Vitek et al., 2009; Gale et al., 2014). This finding holds true in cells isolated from both humans and rodents and in both the periphery and the brain (Gale et al., 2014; Li X. et al., 2015). Therefore, inflammatory triggers such as TBI, infection, and DAMPs produced from metabolic syndrome significantly increase the inflammatory response in APOEε4 carriers. This may potentially lead to the incomplete resolution of inflammation to initiate chronic inflammatory processes that result in neurotoxicity. This trend is evident in HIV-associated dementia, for which E4 carriers have increased risk (Chang et al., 2011).

A heightened inflammatory response occurred in microglia derived from humanized APOEε4 knock-in mice upon on treatment with the TLR3 and TLR4 activator LPS compared to APOEε3 mice (Vitek et al., 2009; Heneka et al., 2015). The inflammatory response was characterized by altered cell morphology, increased nitric oxide production, COX-2 expression, prostaglandin E2 (PGE2) expression, and cytokine production (IL-6, TNF-α, and IL-12p40). In contrast, TREM2 expression was decreased (Vitek et al., 2009; Heneka et al., 2015). A comparable inflammatory response was observed in peripheral macrophages isolated from APOEε4 mice. The E4 allele increases the reactivity of glial and peripheral immune cells, thus aggravating the neurotoxic proinflammatory response (Vitek et al., 2009; Heneka et al., 2015).

DISCUSSION

The etiology of the prodromal phase of AD presents as a complex interplay between several risk factors, which is relevant to therapeutic interventions and preventive strategies (see **Figures 5**, **6**). This implies that therapeutic strategies should employ stratification of patient populations regarding parameters

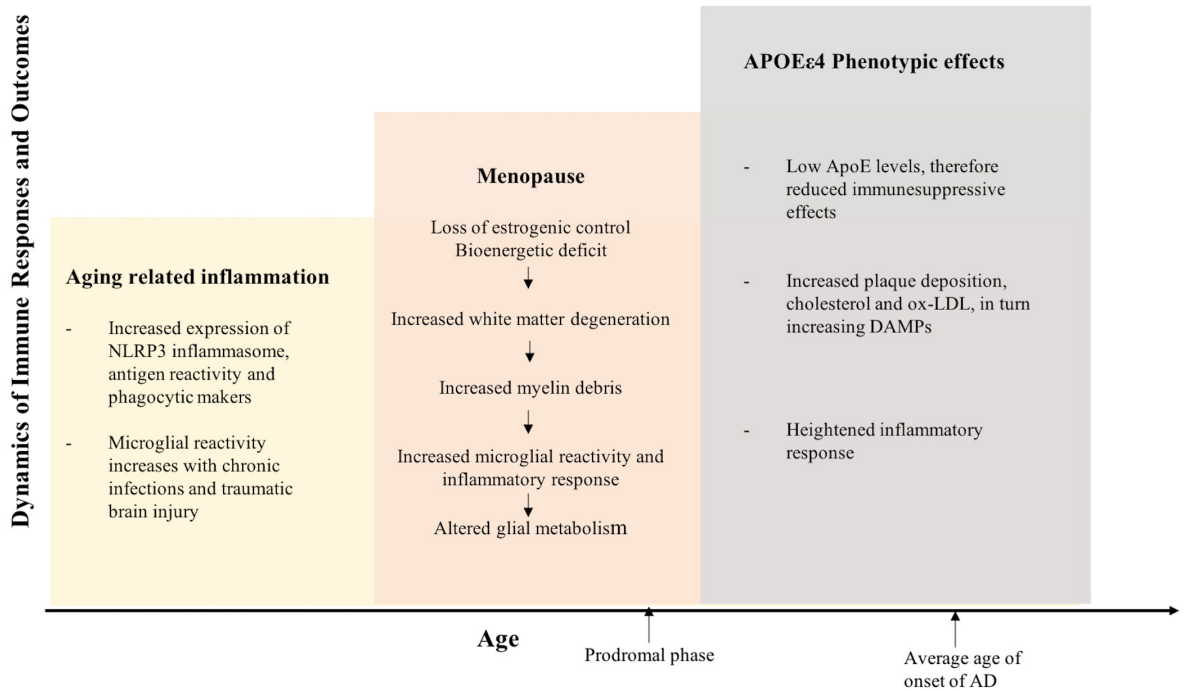

FIGURE 5 | Inflammation integrates Alzheimer's disease risk factors of female sex, chronological age, endocrine aging, and APOEε4 genotype. The three hit model of Alzheimer's risk: aging, menopause, and APOEε4 genotype collectively induce a compromised bioenergetic system in brain that is impacted by the chronic low grade innate inflammation of aging coupled with APOEε4 dysregulated cholesterol homeostasis lead to activation of the adaptive immune response.
The inflammatory immune response is the factor that bridges across each of the risk factors for AD. Immune system regulators that are specific to stage of disease and inflammatory phenotype would provide a therapeutic strategy to disconnect the bridge that drives disease.

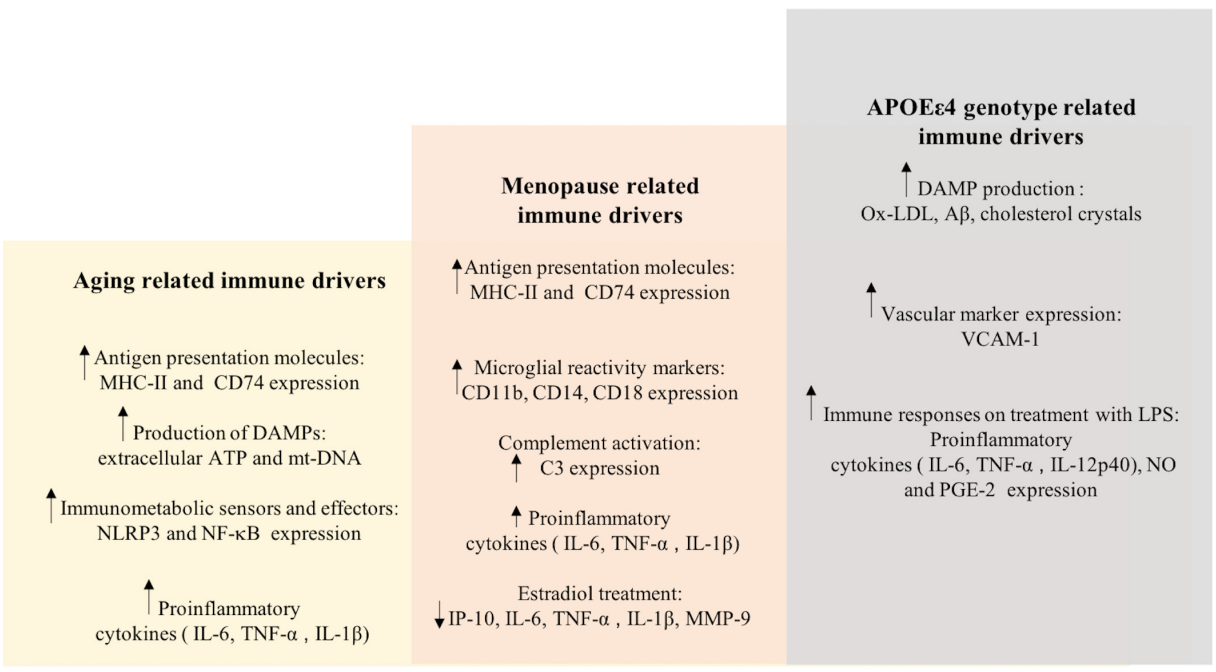

FIGURE 6 | Immune drivers involved in aging, menopause, and APOEε4 genotype related inflammation. Key immune drivers contributing to inflammation due to aging, menopause and APOEε4 genotype related inflammation are detailed to give a global picture of immune dynamics (upward and downward arrows indicate increased and decreased expression, respectively).

of age, sex, and APOE genotype. It also calls for the use of combination therapies that modulate inflammation, lipid-based metabolism in APOEε4 carriers, and loss of estrogenic control in menopausal women. For example, preventive strategies to reduce age-related inflammation could include a combination of NSAIDs and statins in middle-aged adults (45–55 years). In women, this therapy could be modified to include HT during their perimenopausal transition. Patient's medical histories and electronic health records are a source of indicators for chronic inflammation. These strategies should be tailored to the patient's metabolic profile, genetic history, and endocrine-related transition states (see **Figures 5**, **6**).

This understanding of the disease progression also calls for change in design of clinical trials that target the amyloidogenic pathway and treat later stages of the disease pathogenesis. Trial design should incorporate the identification of persons with an increased risk of developing AD, and utilize a risk-factor-based responder analysis. Inflammation-mediated therapeutic and preventive strategies will largely depend upon this stratification of patient populations.

The inflammatory response is influenced by age, chromosomal sex, endocrine transition – menopause and APOE genotype. Inflammation is characteristic of each of these modifying factors and can be a driving force for development of AD. Thus, inflammation has been a therapeutic target in multiple clinical trials for AD. However, each of these trials have failed to meet primary endpoints. Reviewed herein is a consideration of the multiple factors that contribute to and modify the inflammatory phenotype. Going forward, in both discovery and clinical science, it will be important to delineate the etiology of the inflammatory response, the stage of the inflammatory cascade, and the activated network of inflammatory signaling. Inflammation is a moving target and thus requires a precision approach to identifying etiology, stage and appropriate therapeutic target.

CONCLUDING REMARKS

In summary, neuro-inflammatory processes are evident early in the latent prodromal phase and worsen during the course of the Alzheimer's. Disparate results from epidemiological and clinical trials targeting inflammation, highlight the complexity of the inflammatory process. The inflammatory processes that occur during aging, midlife endocrine transitions, and in the APOEε4 carrier contribute to risk and progression of AD. The chronic inflammatory processes that are activated during midlife chronological and endocrine aging, ultimately limit the clearance capacity of microglia and lead to immune senescence. The loss of estrogenic control of bioenergetic function in the brain coupled with dysregulated lipid metabolism in the APOEε4 genotype adversely impact microglial function and clearance mechanisms. The dynamic and context specific activation pattern of the inflammatory processes provide plausible mechanisms underlying failed clinical trials of anti-inflammatory agents in Alzheimer's patients. Collectively, these considerations highlight the rationale for stratifying AD clinical trial cohorts based on their inflammatory phenotype. Combination therapies that include targeted use of anti-inflammatory agent's specific to the immune phenotype could have a higher probability of successfully modifying risk and progression of Alzheimer's disease.

AUTHOR CONTRIBUTIONS

AM and RB wrote and reviewed the manuscript.

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ORIGINAL RESEARCH
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Protective Effect of Hydrogen on Sodium Iodate-Induced Age-Related Macular Degeneration in Mice

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Oxidative stress is one of the main causes of AMD. Hydrogen has anti-oxidative stress and apoptotic effects on retinal injury. However, the effect of hydrogen on AMD is not clear. In this study, fundus radiography, OCT, and FFA demonstrated that HRW reduced the deposition of drusen-like structures in RPE layer, prevented retina from thinning and leakage of ocular fundus vasculature induced by $NaIO_3$. ERG analysis confirmed that HRW effectively reversed the decrease of a-wave and b-wave amplitude in $NaIO_3$-mice. Mechanistically, HRW greatly reduced the oxidative stress reaction through decreased MDA levels, increased SOD production, and decreased ROS content. The OGG1 expression was downregulated which is a marker of oxidative stress. Involvement of oxidative stress was confirmed using oxidative stress inhibitor ALCAR. Moreover, oxidative stress reaction was associated with expression of Sirt1 level and HRW significantly inhibited the downregulation of Sirt1 expression. This result was further confirmed with AICAR which restore Sirt1 expression and activity. In addition, $NaIO_3$-induced retinal damage was related to apoptosis via caspase 8 and caspase 9, but not the caspase 3 pathways, which led to upregulation of Bax and p53, downregulation of Bcl-2, and increase in Jc-1-positive cells in mice. However, HRW effectively reversed these effects that apoptosis induced. These results suggest that HRW protects retinal functions against oxidative stress injury through inhibiting downregulation of Sirt1 and reducing retinal apoptosis. Therefore, we speculated that hydrogen administration is a promising treatment for AMD therapy.

Keywords: hydrogen, sirt1, oxidative stress, apoptosis, AMD

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside; ALCAR, acetyl-L-carnitine; AMD, age-related macular degeneration; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; Bax, BCL2-associated X protein; Bcl-2, B-cell lymphoma-2; DHE, dihydroethidium; ECL, enhanced chemiluminescence; ERG, electroretinography; FFA, fundus fluorescein angiography; FOXOs, Forkhead box protein Os; H&E, hematoxylin and eosin; HRW, hydrogen-rich water; Jc-1, mitochondrial membrane potential assay kit with JC-1; LSD, least significant difference; MDA, malondialdehyde; $NaIO_3$, sodium iodate; OCT, optical correlation tomography; OGG1, 8-oxoguanine DNA glycosylase; PBS, phosphate buffer saline; PCG1-α, receptor γ coactivator-1 α; PVDF, polyvinylidene difluoride membrane; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; Sirt1, silent information regulator 2 homolog 1; SOD, superoxide dismutase; TUNEL, TdT-mediated dUTP Nick-end labeling; VEGF, vascular endothelial growth factor.

INTRODUCTION

AMD is the leading cause of blindness in the elderly population (Syed et al., 2012). The prevalence of AMD gradually increases with age. About 11 million people in the America suffer from visual impairment due to AMD, with approximately 170 million people in the worldwide (Pennington and DeAngelis, 2016). AMD has also become an important cause of blindness in China due to the aging population. The pathogenesis of AMD is related to many factors, such as metabolic disorders, immunity, inflammation, oxidative stress, and so on. However, oxidative stress is one of the main causes of AMD (Hernández-Zimbrón et al., 2018). During the past few decades, although aggressive and combined treatment regimens, including drugs targeting VEGF receptors, laser coagulation, gene therapy, anti-oxidants, and so on, have been used, the rate of blindness is still increasing (Holz et al., 2014; Rakoczy, 2017; Hernández-Zimbrón et al., 2018). Therefore, developing novel therapeutic agents with less toxicity and understanding their molecular mechanisms are necessary for improving AMD outcomes.

Hydrogen as a reducing agent has therapeutic effects for the reduction of oxidative stress, inflammation, and apoptosis (Xie et al., 2012; Xin et al., 2014; Shao et al., 2016; Lin et al., 2017). For example, hydrogen improves oxidant stress-induced organic injury, including acute kidney injury (Du et al., 2016), acute hepatic failure (Sun et al., 2011), chronic obstructive pulmonary disease (Liu Z. et al., 2017), and spinal cord injury (Ge et al., 2017) in rodents. Moreover, it can delay the progress of some diseases, such as diabetes and hypertension (Takeuchi et al., 2015; Guo et al., 2017). In the eye, several laboratories have shown that hydrogen exerts a neuroprotective effect on retinal ganglion cells and on retinal injury induced by light (Tian et al., 2013) in mice. For AMD studies, $NaIO_3$ is used to induce oxidative stress and results in retinal function injury to mimic the characteristics of clinical AMD (Zieger and Punzo, 2016; Berkowitz et al., 2017). However, it remains unclear whether hydrogen has similar effects on AMD.

Sirt1 is a nicotinamide adenine dinucleotide-dependent protein deacetylase, which has been frequently reported to be involved in neuroprotection, cell apoptosis, cell senescence, oxidative stress, and other processes by deacetylating downstream targets (Balaiya et al., 2017). Sirt1 regulates a variety of transcription factors, including p53, FOXOs, PCG1-α, and so on (Luo et al., 2001; Brunet et al., 2004). Moreover, Sirt1 is also considered a longevity molecule to prevent against age-related diseases (Tissenbaum and Guarente, 2001; Bjørklund et al., 2018). In the eye of the rodent, immunostaining from several laboratories showed Sirt1 expression in the outer nuclear layer, inner nuclear layer, and ganglion cell layer of the retina (Jaliffa et al., 2009). Activation of Sirt1 promotes the resistance of neurons to oxidative stress and blocks damage to retinal neurons (Zeng et al., 2016). Additionally, there is more recent experimental evidence indicating that Sirt1 can directly bind to p53 to promote cell survival under stress by specifically repressing the p53-dependent apoptotic response (Brunet et al., 2004). Thus, the activation of Sirt1 may have a beneficial retinal protective effect by reducing intracellular oxidative stress and apoptosis (Zheng and Lu, 2016; Hou et al., 2017; Li et al., 2017a,b; Yao et al., 2018).

The purpose of this study was to investigate the protective role of hydrogen in AMD mice. We observed mouse fundus, retinal structure and function, as well as Sirt1 expression following hydrogen administration. We also tested the hypothesis that hydrogen can regulate retinal oxidative stress reactions and apoptosis pathways. These results suggest that hydrogen is promising treatment in AMD therapy.

MATERIALS AND METHODS

HRW Production

Briefly, HRW was obtained by placing a metallic magnesium stick into drinking water [$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$] and hydrogen final concentration was 0.55∼0.65 mM. The magnesium stick contained 99.9% pure metallic magnesium and natural stones in a polypropylene and ceramic container (Nakao et al., 2010).

Animals

Healthy 8- to 10-week-old C57BL/6 male mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were maintained in a specific pathogen-free grade animal facility under a 12-h light–dark cycle. All procedures were approved by the Committee on Animal Research of Jinzhou Medical University and followed the ARRIVE guidelines pertaining to animal experimentation. $NaIO_3$ (Macklin, Shanghai, China) as an inorganic substance and induced retinal damage is currently recognized as an ideal animal model for AMD research (Kannan and Hinton, 2014). The tail vein injection of $NaIO_3$ to mice can cause oxidative stress reaction in the retina, which induces mice macular degeneration, which is similar to clinical AMD (Wang et al., 2014; Chowers et al., 2017).

Mice were randomly divided into three groups: control group, $NaIO_3$ group, and hydrogen group. Mice in the hydrogen group were given HRW (0.1 ml/g/day, intragastric administration three times daily) by gavage for 7 days prior to a tail vein injection with $NaIO_3$ at a dose of 20 mg/kg, and then HRW treatment continued for 5 days. The vehicle-treated control mice received an equal volume of 0.9% physiological saline.

ALCAR is an inhibitor of oxidative stress (Morigi et al., 2015). To further confirm that hydrogen protects against retinal damage via inhibiting $NaIO_3$-induced oxidative stress, we injected intraperitoneally with ALCAR. In addition, AICAR acts as an agonist of AMPK, which activates Sirt1 after AMPK activation (Tao et al., 2017). To further confirm that hydrogen increases the expression of Sirt1 and protects against $NaIO_3$-induced retinal damage, we injected intraperitoneally with AICAR in $NaIO_3$ mice. So, another group of mice was randomized and pretreated with HRW for 7 days prior to an intraperitoneal injection of ALCAR (0.2 mg/g; Abcam, Cambridge, MA, USA) or AICAR (0.5 mg/g; Abcam), as described in previous studies (Morigi et al., 2015). Mice were then given a tail vein injection of $NaIO_3$

(20 mg/kg) followed by continued HRW administration for 5 days.

Fundus Photography and OCT Examination

After anesthesia with pentobarbital sodium (65 mg/kg) (Yang et al., 2012), fundus photography images were obtained using a retinal imaging system (MicrolV, Phoenix, AZ, United States). OCT images centered on the optic papilla were acquired from anesthetized animals using an OCT system (ISOCT, Optoprobe, Canada).

FFA Assay

After anesthesia with pentobarbital sodium (65 mg/kg), sodium fluorescein (6 mg/kg) solution was injected intraperitoneally (Chung et al., 2017), and tropicamide was used. FFA examination was completed using an imaging system (OPTP-RIS, Optoprobe, Canada).

ERG Analysis

After 12 h of dark adaptation, mice were anesthetized with pentobarbital sodium (65 mg/kg), corneal surface anesthesia was completed with oxybuprocaine hydrochloride eye drops, and tropicamide was used to dilate the pupil. Mice were placed on the operating table. A circular corneal electrode was placed on the surface of the bilateral cornea of the mouse, and a needle-shaped stainless-steel reference electrode was inserted subcutaneously behind the ear of the mouse. A needle-shaped ground electrode punctured the subcutaneous end of the mouse tail (Zhou et al., 2014). The above operation was performed under dark red light. After the baseline stabilized on the display screen, the amplitude changes of the a-wave and b-wave of 3.0 cd.s.m^{-}2 ERG were recorded (ICR, Chongqing, China).

Western Blot Analysis

Tissue homogenates were prepared from retinas of each group after adding the tissue cleavage solution and centrifugation for 30 min at 4°C and 12,000 × *g*. The protein concentration was measured quantitatively with a BCA protein assay kit (P0010s; Beyotime; Shanghai, China). Equal amounts of protein (2 mg/ml) were separated on a 10% SDS-PAGE with an electrophoresis system. Then, the proteins were transferred to a PVDF membrane after electrophoresis and blocked with 1% fetal bovine serum and incubated in strips at 4°C with the following primary antibodies: anti-Sirt1 (ab12193; 1:1000; Abcam), anti-OGG1 (15125-1-AP; 1:1000; Proteintech; Chicago, IL, United States), anti-caspase 3 (ab44976; 1:1000; Abcam), anti-caspase 8 (ab25901; 1:1000; Abcam), anti-caspase 9 (9509S; 1:1000; CST; Massachusetts, United States), anti-P53 (ab131442; 1:1000; Abcam), anti-Bax (ab32503; 1:1000; Abcam), anti-Bcl-2 (ab32124; 1:1000; Abcam), PTG mouse anti-actin (66009-1-Ig; 1:2000; Proteintech; Chicago, IL, United States), and then with anti-rabbit and anti-mouse secondary antibodies (SA00001-2; SA00001-1; 1: 2000; Proteintech) for 2 h at room temperature. ECL emission (1705060; Bio-rad; Hercules, CA, United States) was used to visualize bands, and images were recorded through a gel imaging system. Images were scanned with ImageJ 4.0 software.

Detection of ROS Production in the Retina

ROS levels were measured using a DHE kit (s0063, Beyotime), according to previous studies (Song et al., 2016). Briefly, mouse eyeballs were fixed and then OCT-embedded. Frozen sections (5 μm) were incubated with DHE dye in a 37°C incubator for 30 min. DHE was incubated with a superoxide anion that results in conversion to the red fluorescent compound ethidium. Fluorescence microscopy under the same exposure conditions was used to observe retinal layer ROS content. In retinal sections, the percentage of the ROS area stained with red fluorescence was normalized to the total area examined and quantified with ImageJ 4.0 analysis software.

Jc-1 Detection of Mitochondrial Membrane Potential

The Jc-1 kit (c2006, Beyotime) was used for the detection of the mitochondrial membrane potential, according to a previous study (Mitter et al., 2014). Briefly, frozen tissue sections were incubated with a mixture of Jc-1 stain at 37°C for 20 min in an incubator. The membrane potential of the retina was measured using the same exposure conditions with a fluorescence microscope. The percentage of the apoptosis area stained with green fluorescence was normalized to the total area examined and quantified with ImageJ 4.0 software.

Oxidative Stress Levels Detected

Retina tissues were weighed and washed in PBS and then homogenized immediately in 10 volumes of PBS at 37°C. After centrifugation, supernatants were collected and stored at −80°C. The levels of activated SOD and oxidative stress product MDA were measured according to the instructions of the kit (A001-3, A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was read on a microplate reader (Denley Dragon, Wellscan MK3, Thermo, Finland), and the concentrations were calculated based on a standardized curve.

Statistical Analysis

All data are expressed as the mean ± SEM, and one-way ANOVA was used to compare differences between the groups. The LSD test was used to compare multiple pairs of means. Comparison between two groups was done with a *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

HRW Has Protective Effects on $NaIO_3$-Induced Retinal Damage

To assess the protective effect of HRW treatment on $NaIO_3$-induced retinal damage in mice, we performed eye functional examinations in $NaIO_3$ mice with or without HRW treatment. Fundus photography revealed a large number of yellow-white,

drusen-like structures in the fundus of $NaIO_3$-treated mice. We observed a significant reduction in yellow-white deposits on the fundus after HRW treatment (**Figure 1A**). By OCT, we found that the RPE layer of mice in the $NaIO_3$ group developed a large number of high reflex zones and the retina became thin (**Figure 1B**). However, HRW prevented retinal thinning and reduced the number of high reflex zones in the RPE layer in mice that received HRW-treatment (**Figure 1C**, $P < 0.01$). To further confirm the damage of $NaIO_3$ to the retina, we examined the retinal vascular integrity. The results of the FFA assay showed that retinal blood vessel leakage appeared in $NaIO_3$-treated mice (**Figure 1D**), but the retinal leakage area in HRW-treated mice was much smaller than that in $NaIO_3$ mice (**Figure 1E**, $P < 0.01$). These results confirm the protective effect of HRW on retinal injury.

Studies have shown that $NaIO_3$ can damage RPE cells and retinal visual function (Zieger and Punzo, 2016). We assessed the effect of HRW on retinal function using an ERG assay in mice. The a-wave reflects the function of the cone cells and the rod cells, and the b-wave represents the function of the bipolar cells. In the dark-adapted 3.0 ERG, a- and b-wave amplitudes were significantly increased in the HRW-treated mice compared to those in the $NaIO_3$ mice, reflecting the recovery of visual function in HRW-treated mice and the resistance to $NaIO_3$-induced functional visual damage by HRW (**Figure 2**; $P < 0.05$). This result suggested that HRW can protect the retina from $NaIO_3$-induced damage in mice.

HRW Reduces the Oxidative Stress Reaction in the Mouse Retina After $NaIO_3$ Treatment

The mechanism of $NaIO_3$-induced retinal damage is associated with oxidative stress, but whether the protective effect of HRW on the retina is through a reduction in oxidative stress was examined. First, we detected $NaIO_3$-induced retinal damage with H&E staining. It clearly showed that a large amount of melanin deposition appears in the RPE layer retinas from $NaIO_3$ mice compared to retinas from control mice. The amount of the black sediment on the retina in HRW-treated mice is greatly reduced compared to the amount in $NaIO_3$ mice (**Figure 3A**). Moreover, the retinal layers in $NaIO_3$ mice were thinner than those of the control mice. However, HRW prevents retinal thinning (**Figure 3B**, $P < 0.01$). In addition, the thickness of the outer nuclear layer in $NaIO_3$ mice is significantly reduced compared to

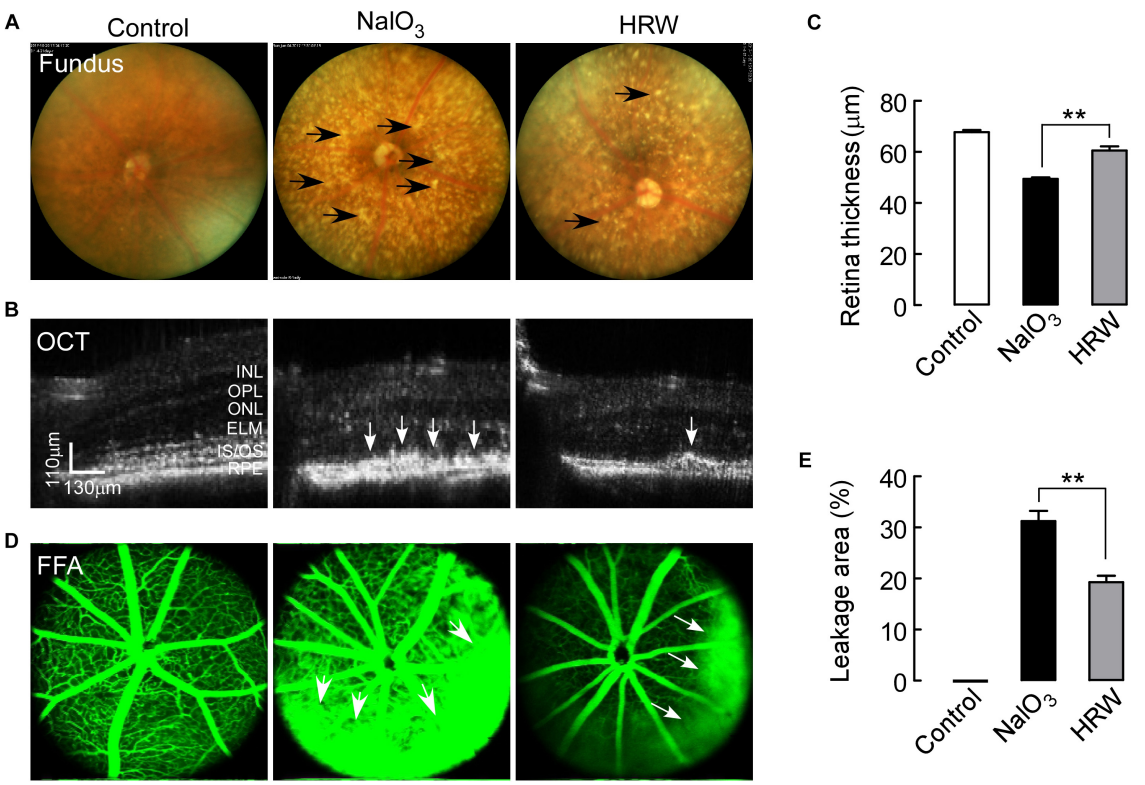

FIGURE 1 | Effects of HRW on $NaIO_3$-induced fundus and retinal impairment in mice. After cotreatment with HRW (0.1 ml/g/day, i.g.) and $NaIO_3$ (20 mg/kg, i.v.) for 12 days, retinas were evaluated with the fundus photography, OCT examination, and FFA examination. **(A)** Representative images of fundus photography. The black arrow represents yellow-white, drusen-like structures. **(B)** Representative images of OCT in the mouse retina and **(C)** quantification of retinal thickness. White arrows indicate areas of hyperreflexia in the RPE area. **(D)** Representative images of FFA and **(E)** quantification of the leakage area. White arrowheads represent the leaking area. RPE, retinal pigment epithelium; IS/OS, inner segment/outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Values are presented as the mean ± SEM, $n = 5$, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

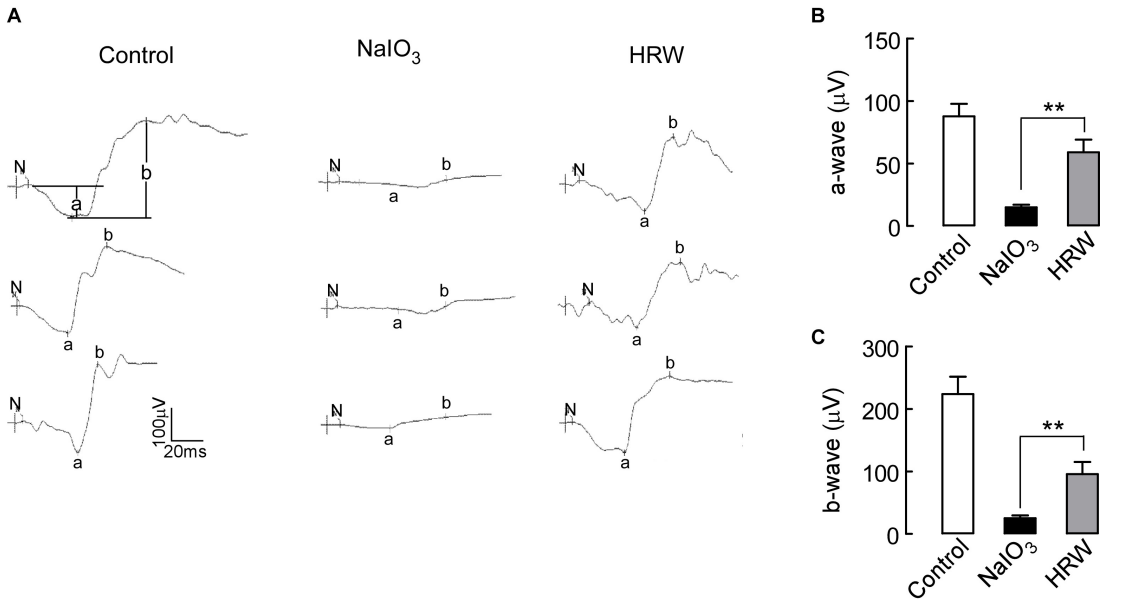

FIGURE 2 | Effects of HRW on $NaIO_3$-induced visual function impairment in mice. **(A)** ERG map of dark adaptation 3.0 in control, $NaIO_3$, and HRW groups. N represents the start of stimulation, a represents the amplitude of the a-wave, and b represents the b-wave amplitude. **(B,C)** Quantification of the a-wave and b-wave amplitudes, respectively. Values are presented as the mean ± SEM, $n = 10$, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

that in control mice (**Figure 3C**, $P < 0.01$), which indicated that $NaIO_3$ caused retinal injury. In addition, we assessed the toxic effect of $NaIO_3$ on the liver using H&E staining. No changes were observed in liver sections between the three groups (**Figure 3D**), suggesting that $NaIO_3$ selectively damages the eyeball.

Next, we observed the oxidative stress reaction in the retina. As **Figure 4A** shows, HRW significantly increased the activity of the oxidative stress inhibitory enzyme SOD, whereas it decreased MDA content in $NaIO_3$ mice. To further confirm this result, we examined the expression level of OGG1, an oxidative stress marker protein, in the retina. The expression of OGG1 in $NaIO_3$ mice is greatly increased compared to control expression. However, HRW significantly reduced the $NaIO_3$-induced increase in OGG1 expression (**Figure 4B**; $P < 0.05$). DHE staining, which reflects the ROS content in the retina, showed a marked decrease in red fluorescence of ROS-stained retinal tissue in HRW-treated mice compared to the retinas of $NaIO_3$ mice (**Figure 4C**; $P < 0.01$). These results suggest that HRW can attenuate the oxidative stress reaction by $NaIO_3$-induction in the mouse retina.

HRW Inhibits Downregulation of Oxidative Stress-Induced Sirt1 in Mice

Sirt1 plays an important role in various retinal diseases, including anti-oxidant and anti-apoptotic effects in mice (Han et al., 2017). In this study, we assessed whether HRW reduced the oxidative stress reaction through the regulation of Sirt1 expression. Western blotting indicated that Sirt1 expression was significantly downregulated in $NaIO_3$ mice, and HRW treatment reversed the $NaIO_3$-induced downregulation of Sirt1 expression (**Figure 5A**; $P < 0.05$).

We next tested whether HRW inhibits the downregulation of Sirt1 through a reduction in oxidative stress. Mice were injected with ALCAR, an inhibitor of oxidative stress, before $NaIO_3$ treatment, and then we measured Sirt1 and OGG1 expression. We found that ALCAR significantly reduced the $NaIO_3$-induced downregulation of Sirt1 and increased OGG1 expression in the mouse retina (**Figure 5B**; $P < 0.01$). These results confirm that HRW indeed inhibits the downregulation of Sirt1 expression by decreasing oxidative stress.

In addition, we further analyzed whether HRW protects $NaIO_3$-induced retinal damage through the regulation of Sirt1 expression. Mice were treated with the Sirt1 indirect activator AICAR prior to $NaIO_3$ administration. We found that AICAR treatment significantly increased the expression of Sirt1 in $NaIO_3$ mice (**Figure 5C**; $P < 0.01$). At the same time, we measured the expression of OGG1. AICAR could also inhibit the $NaIO_3$-induced OGG1 upregulation (**Figure 5C**; $P < 0.01$), suggesting that HRW could inhibit the downregulation of oxidative stress-induced Sirt1 expression.

HRW Inhibits $NaIO_3$-Induced Apoptosis Through the Regulation of Sirt1 Expression in the Mouse Retina

Studies have shown that $NaIO_3$ induces retinal damage by activating the pathway of apoptosis (Jaliffa et al., 2009; Balmer et al., 2015; Zeng et al., 2016). We first examined apoptosis in the retina of $NaIO_3$ mice. TUNEL staining showed that TUNEL-positive cells (green) were mainly concentrated in the outer nuclear layer of the retina in the $NaIO_3$ mice. HRW greatly decreased the number of TUNEL-positive cells

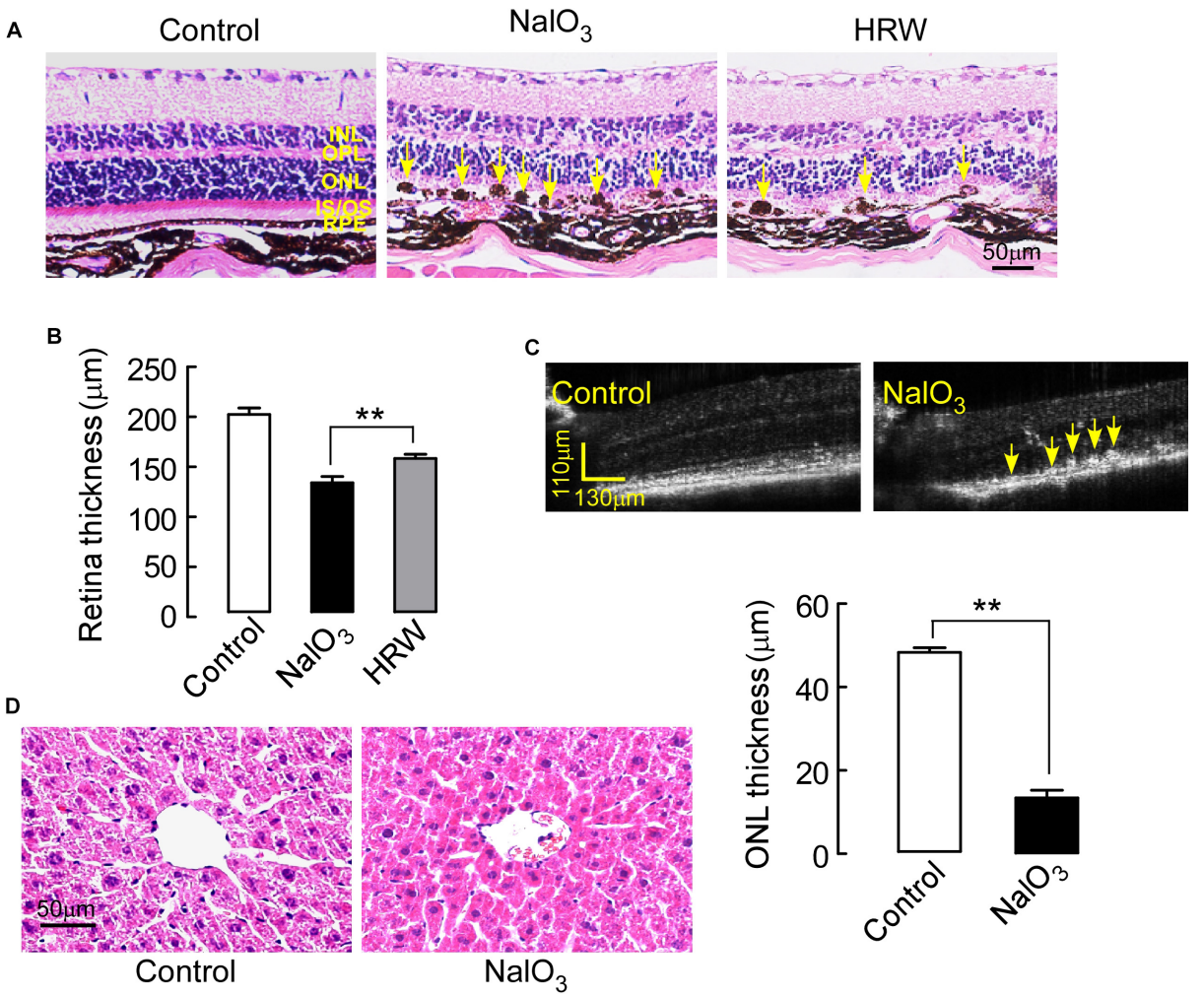

FIGURE 3 | Effects of HRW on $NaIO_3$-induced retinal morphological impairment in mice. Mouse eyeballs were harvested after cotreatment with HRW and $NaIO_3$ for 12 days. **(A)** Representative images of H&E-stained eyeball sections from control and $NaIO_3$ mice treated with or without HRW. Yellow arrows indicate drusen-like melanin depositions in the RPE layer. **(B)** Quantitative analysis of retinal thickness via H&E-stained eyeball sections. The values are expressed as the mean ± SEM, $n = 10$. **(C)** Representative images of OCT in the mouse retina in control and $NaIO_3$ groups. Yellow arrows point toward areas of hyperreflexia in the RPE. **(D)** H&E staining of the liver after 5 days of $NaIO_3$ injection. $n = 5$, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

in the retina (**Figure 6B**; $P < 0.01$). Further, we analyzed the expression of apoptosis pathway-related proteins in the mouse retina. However, no change was observed in the expression of caspase 3 between mice in all groups ($P > 0.05$). HRW significantly reduced the expression of caspase 8 ($P < 0.01$), caspase 9 (**Figure 6A**; $P < 0.01$), p53, and Bax, whereas it increased Bcl-2 expression (**Figure 6C**; $P < 0.01$) in $NaIO_3$ mice. Additionally, Jc-1 can be used to detect early apoptosis by indicating changes in the mitochondrial membrane potential (Wang et al., 2015). As shown in **Figure 6D**, early apoptotic cells (green) are abundantly apparent in the retina of $NaIO_3$ mice, and the green fluorescence intensity of Jc-1-staining in HRW-treated mice is significantly decreased ($P < 0.01$). These results suggest that HRW could inhibit $NaIO_3$-induced retinal apoptosis via caspase 8 and caspase 9 apoptotic pathways.

Sirt1 has anti-apoptotic and anti-oxidative stress effects in the rat (Yang et al., 2013; Qi et al., 2015). To demonstrate the anti-apoptotic effect of Sirt1, we examined the expression of apoptosis-related regulators after administration of the Sirt1 indirect agonist AICAR in mice. AICAR significantly inhibited the expression of caspase 8, caspase 9, p53, and Bax, but increased the expression of Bcl-2 in $NaIO_3$ mice (**Figure 6E**; $P < 0.01$). These results confirm that HRW has an anti-apoptotic effect through the increase of Sirt1 expression in $NaIO_3$-induced retinal damage.

In addition, we further determined the relationship between oxidative stress and apoptosis using an inhibitor of oxidative stress in $NaIO_3$ mice. After mice were given ALCAR, we examined the expression of proapoptotic proteins (caspase 8, caspase 9, p53, and Bax) and the anti-apoptotic protein Bcl-2. ALCAR significantly inhibited the upregulation of proapoptotic

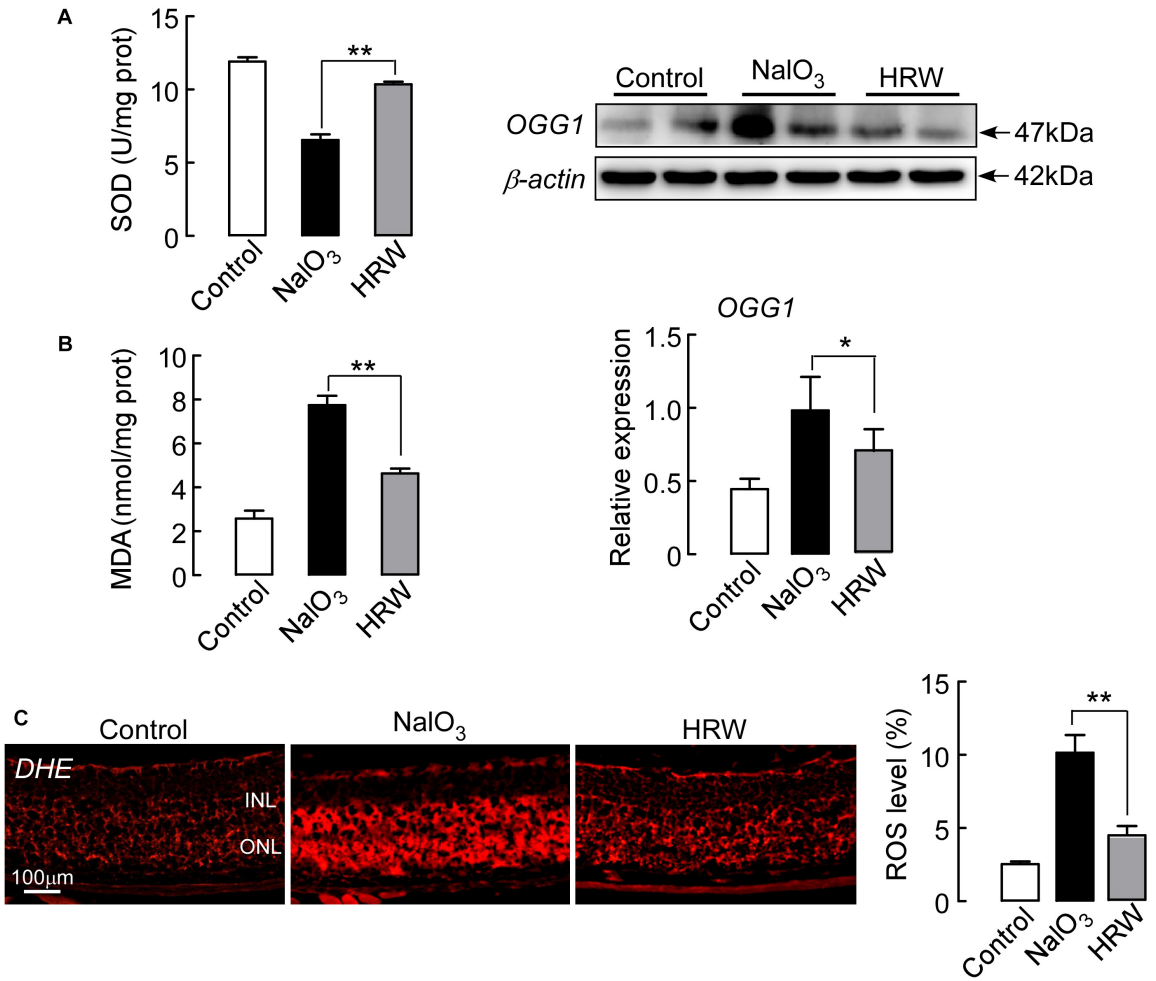

FIGURE 4 | Effect of HRW on $NaIO_3$-exerted retinal oxidative stress in mice. Retinal tissue collection after cotreatment with HRW and $NaIO_3$ for 12 days. **(A)** The level of SOD and MDA in the retina was analyzed by SOD and MDA kits. **(B)** Representative blots and densitometry data of OGG1 expression in the retinas from $NaIO_3$ mice with and without HRW treatment. **(C)** The activity of ROS was measured by DHE staining, the red staining is positive for ROS content. Data represent the mean ± SEM of three independent experiments, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

protein expression and downregulation of Bcl-2 protein in $NaIO_3$ mice (**Figure 6F**; $P < 0.01$). These results suggest that HRW could effectively inhibit the downregulation of Sirt1 expression through antioxidative stress and has a further antiapoptotic effect on $NaIO_3$-induced retinal injury.

DISCUSSION

Previous studies have indicated that hydrogen has ideal therapeutic effects for oxidant stress and inflammation (Xin et al., 2014; Lin et al., 2017). Although some studies have demonstrated the anti-apoptosis and anti-oxidative stress effects of hydrogen on light-induced retinal injury in the rodent (Takeuchi et al., 2015), the effect of hydrogen on AMD is still unknown. In the present study, we investigated the retinal protective effects and mechanisms of action of hydrogen in $NaIO_3$-induced mice. Our data demonstrated that HRW could reduce retinal damage through a decreased oxidative stress reaction and inhibit the $NaIO_3$-induced downregulation of Sirt1 expression (**Figures 4**, **5A**). Involvement of oxidative stress and the Sirt1 protein was confirmed using the oxidative stress inhibitor ALCAR and the Sirt1 activator AICAR (**Figures 5B,C**). Moreover, HRW inhibits apoptosis via the caspase 8 and 9 pathways, but not the caspase 3 pathway, in $NaIO_3$ mice retinas (**Figure 6**). Interestingly, we found that the injury of $NaIO_3$ is tissue selective and $NaIO_3$ mouse liver sections did not display a significant toxic effect with H&E staining (**Figure 3**).

HRW can improve the quality of human life and prevent the occurrence of diseases (Mizuno et al., 2018). HRW also can protect the retina from injury in eye diseases in rats (Qi et al., 2015; Chen et al., 2016). We investigated the protective effect of HRW in AMD mice. After mice were given HRW and $NaIO_3$, through fundus photography and OCT we found that HRW reduced the deposition of yellow-white, drusen-like structures induced by $NaIO_3$ and prevented the thinning of the retina

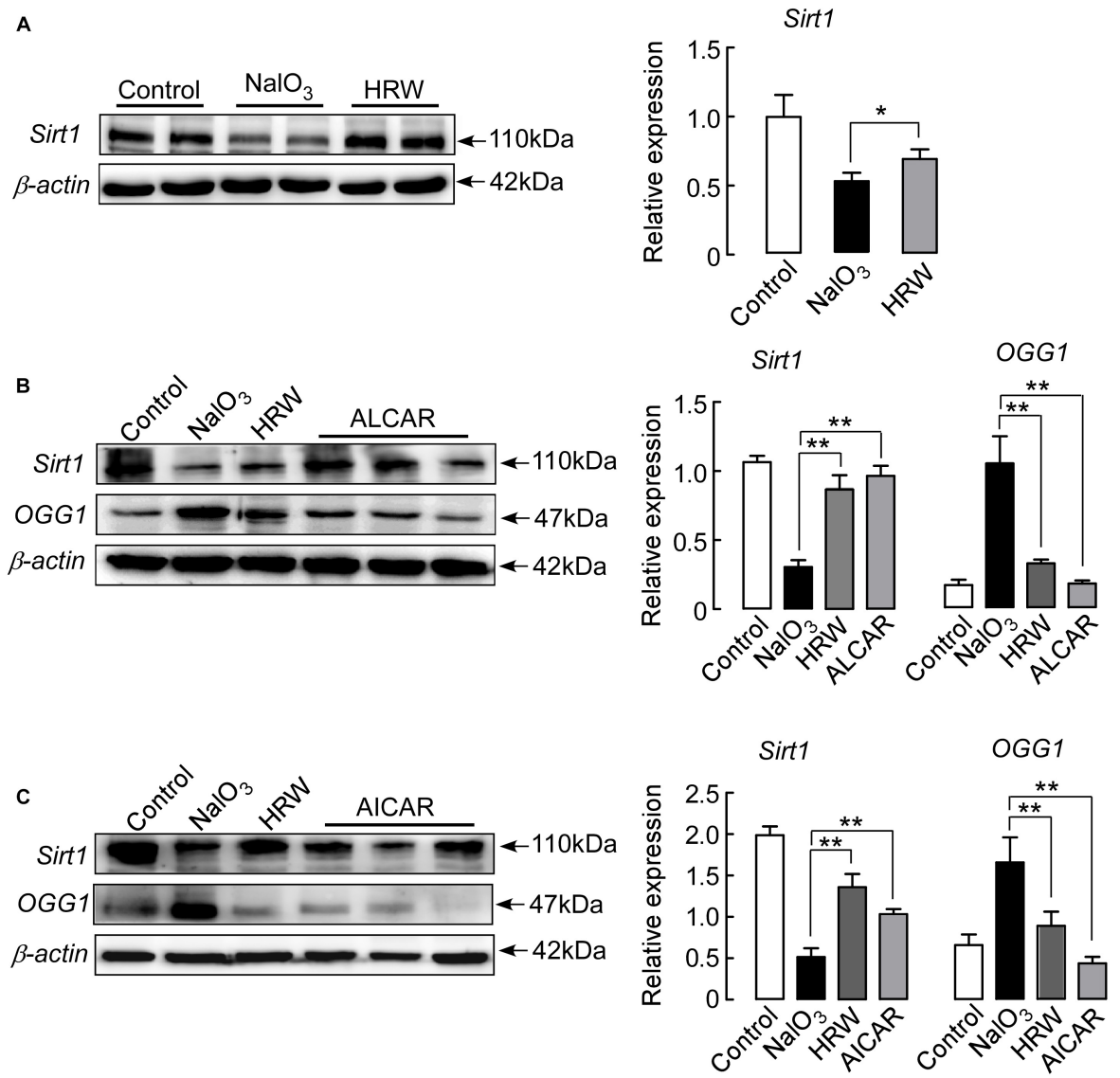

FIGURE 5 | HRW upregulates Sirt1 expression in the retina by inhibiting oxidative stress. **(A)** Representative blots and quantitative analysis of Sirt1 protein expression at 5 days after $NaIO_3$ injection. Mice were pretreated with ALCAR (0.2 mg/g, i.p.) **(B)** or AICAR (0.5 mg/g, i.p.) **(C)** before $NaIO_3$ injection. The expression of Sirt1 and OGG1 were measured by Western blotting. Data represent the mean ± SEM of three independent experiments, $n = 3$–6, $^*P < 0.05$, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

(**Figures 1A,B**). Consistently, it reduced the degree of retinal degeneration. We further found that HRW reduced the area of fundus leakage induced by $NaIO_3$ and maintained the integrity of fundus vessels (**Figure 1D**). Further, when we observed the retinal function by ERG, we found that HRW increased the survival of rod and cone cells and reversed the degree of visual function impairment by $NaIO_3$ (**Figure 2**).

Oxidative stress is one of the many factors in the pathogenesis of AMD (Hernández-Zimbrón et al., 2018). $NaIO_3$ induces retinal injury through oxidative stress (Kannan and Hinton, 2014; Berkowitz et al., 2017). As hydroxyl radicals, hydrogen can combine with excess oxygen free radicals, causing inhibition of the oxidative stress reaction by decreasing MDA content and upregulating SOD (Wu et al., 2017). There is growing evidence that hydrogen plays important roles in ROS reduction (Liu et al., 2014). After administration of $NaIO_3$, the RPE layer of the retina became thinner or even ruptured. There was a large amount of black deposits, similar to drusen, between the RPE layer and Bruch's membrane (**Figure 3A**). The ONL membrane and the overall thickness of the retina were decreased (**Figure 3C**). HRW can reduce retinal morphological damage induced by $NaIO_3$ (**Figure 3B**). The index of oxidative stress showed that HRW decreased the content of MDA in oxidative stress products and prevented the decrease of SOD activity with the oxidative stress inhibitor after $NaIO_3$ treatment (**Figure 4A**). In addition, we observed the amount of ROS induced by $NaIO_3$ was reduced

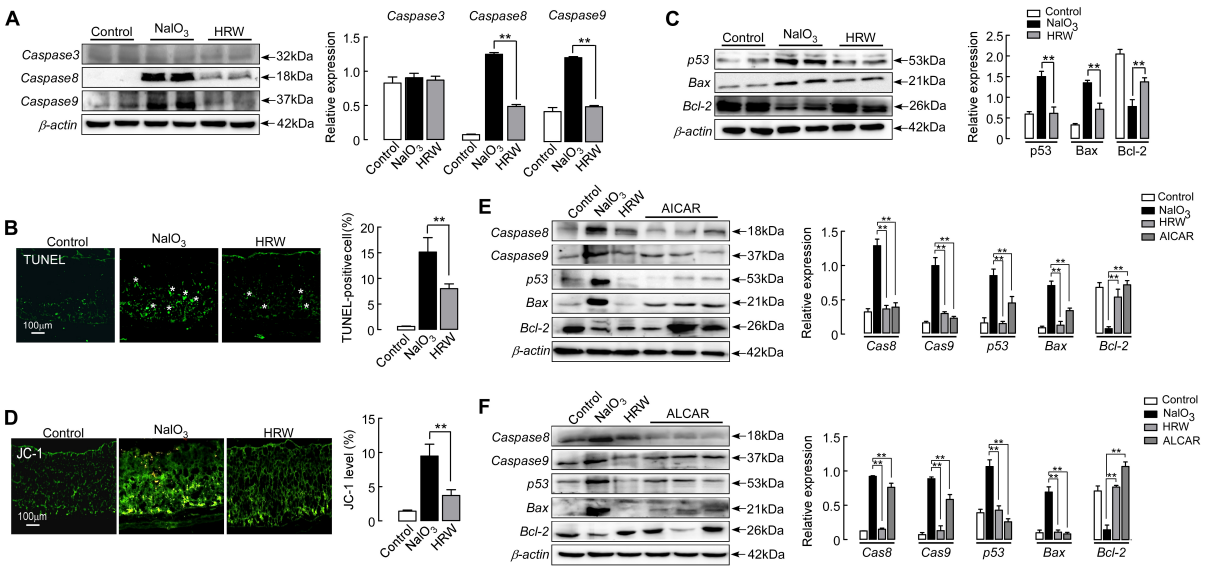

FIGURE 6 | HRW reduces apoptosis in the retina by upregulating Sirt1. **(A)** Representative blot and quantitative analysis of caspase 3, caspase 8, and caspase 9 protein expression levels at 5 days after $NaIO_3$ injection with or without HRW administration. **(B)** Representative images of TUNEL-stained retinal sections from control and $NaIO_3$ mice with or without HRW administration. TUNEL-positive cells are shown by asterisks. Data represent the mean ± SEM of three independent experiments. $n = 10$. **(C)** Representative blot and the quantitative analysis of protein expression for p53, Bax, and Bcl-2 expression in the retina. $n = 4$. **(D)** Representative images of Jc-1-stained retinal sections and the quantification of green fluorescence intensity in retinal sections. $n = 5$. Representative Western blot and the quantitative analysis of caspase 8, caspase 9, p53, Bax, and Bcl-2 protein expression after ALCAR **(E)** or AICAR **(F)** injection in retinas from $NaIO_3$ mice. Values were presented as the mean ± SEM. $n = 3$–6, $^{**}P < 0.01$ vs. the $NaIO_3$ group.

by HRW because there was a decreased number of ROS-positive cell as shown by the fluorescent intensity of DHE staining in the retina (**Figure 4C**). Further, to determine that HRW can inhibit oxidative stress, we measured the expression of the oxidative stress marker protein OGG1. The results of our study showed that hydrogen significantly decreased oxidant levels induced by $NaIO_3$ (**Figure 4B**).

As a deacetylase, Sirt1 is closely related to apoptosis and oxidative stress (Chen et al., 2016). Sirt1 plays a protective role in regulating apoptosis in the retina. With increasing age, the expression of Sirt1 increases in the retina, thus increasing the resistance of the retina to the outside damage (Zeng and Yang, 2015). Hydrogen can activate Sirt1 and protect against damage from diseases, and hydrogen can inhibit oxidative stress and regulate the expression of Sirt1. In our studies, the expression of Sirt1 was downregulated by $NaIO_3$ administration (**Figure 5A**). However, HRW could elevate the expression of Sirt1 by inhibiting oxidative stress (**Figure 5B**). Furthermore, oxidative stress inhibitors and Sirt1 activators caused an increase in Sirt1 levels and decrease in OGG1 levels, suggesting that the antioxidant stress of HRW can prevent the downregulation of Sirt1 induced by $NaIO_3$ (**Figure 5C**). Our study indicated that hydrogen directly mediates the expression of Sirt1 by anti-oxidative stress.

Studies have shown that hydrogen inhibits apoptosis by activating Bcl-2 and inhibiting Bax and caspase 3 (He et al., 2016; Chen et al., 2017). Sirt1 inhibits apoptosis by upregulating the expression of Bax (Liu S. et al., 2017). Sirt1 can inhibit apoptosis by regulating the Bcl-2 family and caspase 3 (Gu et al., 2018). Some studies suggest that oxidative stress inhibits the activity of Sirt1 and then regulates p53-dependent apoptosis (He et al., 2017). In this study, we used TUNEL staining to demonstrate that HRW has an anti-apoptotic effect (**Figure 6B**). HRW also reduced the expression of caspase 8 and caspase 9 but did not affect caspase 3 expression (**Figure 6A**). Moreover, our studies further found that HRW decreased the expression of p53 and Bax, but increased the expression of Bcl-2 after $NaIO_3$ administration (**Figure 6C**), suggesting that the anti-apoptotic effect of hydrogen was through the caspase 8 and caspase 9 pathways. The measurement of the mitochondrial membrane potential also confirmed the role of the anti-apoptotic effect of HRW (**Figure 6D**). The HRW inhibition of apoptosis was mimicked by oxidative stress inhibitors and Sirt1 activators (**Figures 6E,F**). These results suggest that hydrogen could promote cell survival in the retina after oxidative stress, and hydrogen inhibits apoptosis by regulating the expression of Sirt1. Our results suggest that hydrogen inhibits oxidative stress and the downregulation of Sirt1 induced by $NaIO_3$ and plays a role in preventing apoptosis.

Molecular hydrogen as a medical gas could be used in antioxidant therapy in numerous human diseases (Kurokawa et al., 2015). The primary advantage of HRW is that safe means of delivering hydrogen (Chen et al., 2016). Hydrogen is a colorless, transparent, odorless, and tasteless gas. It is insoluble in water as the lightest gas. *In vitro* experiments were carried out under normal pressure. Because of the characteristics of hydrogen and the limitations of our current laboratory conditions, in our

studies, AMD animal model was used to confirm the therapeutic effect of hydrogen on AMD. At the same time, we used oxidative stress inhibitors and Sirt1 activators to confirm the role of hydrogen in anti-oxidative stress and anti-apoptosis of AMD. We will conduct *in vitro* experiments if conditions permit to further support our point of view.

In summary, we found that hydrogen can reverse the production and progress of drusen, improve the function of the optic nerve and the integrity of fundus vessels. Hydrogen regulates the expression of Sirt1 in the retina by inhibiting oxidative stress. Hydrogen inhibits the downregulation of Sirt1 induced by $NaIO_3$ and inhibits apoptosis induced by $NaIO_3$ via regulation of Sirt1 expression. Our findings suggest that hydrogen is an effective therapeutic strategy for the pathogenesis of oxidative stress in AMD.

AUTHOR CONTRIBUTIONS

LL and YL conceived and designed the experiments. YL, RL, JH, JX, XH, and FR performed the experiments. LL, YL, RL, and FR analyzed the data. LL contributed reagents, materials, and analysis tools. LL and YL wrote the paper. XH contributed the process of revised article.

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frontiers in Aging Neuroscience

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Revisiting the Role of Insulin-Like Growth Factor-I Receptor Stimulating Activity and the Apolipoprotein E in Alzheimer's Disease

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Background: Alterations in insulin-like growth factor I (IGF-I) signaling have been associated with dementia and Alzheimer's disease (AD). Studies on the association between IGF-I levels and dementia risk have been inconclusive. We reported earlier that higher levels of IGF-I receptor stimulating activity are associated with a higher prevalence and incidence of dementia.

Objective: In the present study, we test the robustness of the association between IGF-I receptor stimulating activity and dementia by extending the follow-up period to 16 years and investigate possible effect modification by apolipoprotein E (ApoE).

Methods: At baseline, circulating IGF-I receptor stimulating activity was determined by the IGF-I kinase receptor activation (KIRA) assay in 1,014 elderly from the Rotterdam Study. Dementia was assessed from baseline (1997–1999) to follow-up in January 2015. Associations of IGF-I receptor stimulating activity and incident dementia were assessed with Cox proportional hazards models.

Results: During 10,752 person-years of follow-up, 174 people developed dementia. In the extended follow-up we no longer observed a dose-response relationship between IGF-I receptor stimulating activity and risk of dementia [adjusted odds ratio 1.11; 95% confidence interval (CI) 0.97–1.28]. Interestingly, we found evidence of an interaction between ApoE-ε4 and tertiles of IGF-I receptor stimulating activity. IGF-I receptor stimulating activity in the median and top tertiles was related to increased dementia incidence in hetero- and homozygotes of the ApoE-ε4 allele, but did not show any association with dementia risk in people without the ApoE-ε4 allele (adjusted odds ratio medium vs. low IGF-I receptor stimulating activity in ApoE-ε4 carriers: 1.45; 95% CI 1.00–2.12). These findings suggest a threshold effect in ApoE-ε4 carriers.

In line with the hypothesis that downregulation of IGF-I signaling is associated with increased dementia risk, ApoE-ε4 homozygotes without prevalent dementia displayed lower levels of IGF-I receptor stimulating activity than heterozygotes and non-carriers.

Conclusion: The findings shed new light on the association between IGF-I signaling and the neuropathology of dementia and ask for replication in other cohorts, using measures of IGF-I receptor stimulating activity rather than total serum levels as putative markers of dementia risk.

Keywords: Alzheimer's disease, dementia, genetic epidemiology, insulin-like growth factor I, KIRA assay, apolipoprotein E

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a multifunctional peptide hormone known to modulate multiple cellular processes including proliferation, differentiation, energy metabolism, glucose homeostasis, stress resistances and apoptosis. Downregulation of IGF-I signaling is found in the elderly and in patients with type 2 diabetes. In contrast, elevated concentrations of circulating IGF-I have been associated with an increased risk of prostate, breast (Hankinson et al., 1998; Renehan et al., 2004; Kaaks, 2008), colorectal (Ma et al., 1999; Wu et al., 2002; Kaaks, 2008) and lung (Yu et al., 1999) cancer. IGF-I signaling is also markedly disturbed in the brain of patients with Alzheimer's Disease (AD; Frölich et al., 1998, 1999; Moloney et al., 2010) with alterations in both the levels and phosphorylation state of IGF-I receptor (IGF-IR) as well as the levels and distribution of IGF-I and IGF-IR mRNA in the brain (Rivera et al., 2005; Steen et al., 2005). Dysregulation progresses as the disease advances (Ostrowski et al., 2016). It remains unclear whether alterations in IGF-I signaling are a causal factor in the pathogenesis of AD or rather a consequence. Findings of experimental and observational studies have been controversial.

In experimental studies, reduced IGF-I signaling has been linked to increased amyloid β (Aβ) deposition (Carro et al., 2002; Ashpole et al., 2015), development of phosphorylated tau (Gasparini et al., 2001, 2002; Cheng et al., 2005), increased oxidative stress, neuro-inflammation and apoptosis (Bedse et al., 2015). IGF-I can increase the transport of Aβ carrier proteins albumin and transthyretin into the brain. Upon systemic administration, brain levels of albumin and transthyretin increased and the fraction of Aβ bound to carrier proteins in the CSF and blood was elevated. Suggesting that IGF-I reduced brain Aβ load, in part by enhancing its clearance through carrier proteins such as albumin and transthyretin (Carro et al., 2002). Systemic administration of IGF-I has also been shown to lower the toxicity of Aβ in wild type mice (Aguado-Llera et al., 2005) and restore cognitive function in mouse models of AD (Carro et al., 2006), supporting the potential of IGF-I as a therapeutic target in human patients. Peripheral administration has, however, failed to alter Aβ levels in trials with transgenic rats, mice and dogs (Lanz et al., 2008; Sevigny et al., 2008; Parrella et al., 2013; Trueba-Sáiz et al., 2013). In contrast to the neuroprotective role of IGF-I, it has also been suggested that the downregulation of IGF-I signaling attenuates the effects of aging and neurodegeneration. Suppression of IGF-I signaling has been associated with longevity in humans (Suh et al., 2008) and has shown to delay the process of aging and increase lifespan in model organisms (Tatar et al., 2001; Tazearslan et al., 2011; Milman et al., 2014). In AD mouse models long term suppression of IGF-IR signaling has been linked to reduced neuronal loss, greater resistance to oxidative stress, neuro-inflammation and Aβ aggregation, and has been associated with prolonged preservation of spatial memory and a reduction of behavioral deficits, even when plasma Aβ levels increased (Cohen et al., 2009; Freude et al., 2009; Gontier et al., 2015; George et al., 2017). Last but not least, lowering serum IGF-I *via* a protein restriction diet ameliorated Alzheimer pathology in transgenic mouse models (Parrella et al., 2013).

In human observational studies the role of IGF-I signaling in the risk of AD and dementia remains open to question. Longitudinal analyses of the cumulative dementia incidence in 3,582 participants of the Framingham Heart Study, spanning middle and old age, indicated that for those with the lowest levels of serum IGF-I at baseline dementia risk was increased by 51% (Westwood et al., 2014). No such relation was found by Green et al. (2014) examining the prospective association between total IGF-I, IGF-II, and IGF-I Binding Protein 3 (IGFBP-3) and cognitive function in 724 males participating in the Caerphilly Prospective Study. In this study, both total serum IGF-II and IGFBP-3 were associated with age-related cognitive decline and cognitive impairment, but previous associations of total serum IGF-I with cognitive decline and dementia were not replicated. Correspondingly, a meta-analysis of epidemiological studies on the association between total serum IGF-I and dementia nullified the results of previous studies. Five studies suggested that increased levels of circulating total IGF-I predict a higher risk of AD, while three studies suggested an inverse association and two studies reported no significant differences between groups (Ostrowski et al., 2016). Differences in findings across studies are speculatively attributed to differences in age of onset and stage of disease progression, comorbid diabetes, or the differential influence of IGF-I gene polymorphisms. Although, the majority of studies report a contribution of alterations in IGF-I signaling to the prediction of dementia risk independent of apolipoprotein E *(ApoE)* genotype (Vargas et al., 2011; Talbot et al., 2012; van Exel et al., 2014; Lane et al., 2017), Deelen et al. (2011) reported

an association between the ApoE-ε4 allele and lowered total serum IGF-I levels in middle-aged women. However, a recent Mendelian randomization study by Williams et al. (2018) did not provide any evidence for an association between genetically predicted variation in total IGF-I or its binding protein IGFBP-3 and risk of AD. These findings decrease the probability that total serum IGF-I is the relevant determinant of AD and dementia.

As most of the circulating IGF-I measured in serum is bound to IGF-I binding proteins and therefore biologically inactive, levels of total IGF-I poorly reflect the actual IGF-I bioactivity. We therefore applied an IGF-I specific kinase receptor activation assay (KIRA) to assess IGF-I bioactivity, by measuring IGF-I receptor stimulating activity (Chen et al., 2003; Brugts et al., 2010). IGF-I receptor stimulating activity takes into account the modifying effect of IGF-I binding proteins on the interaction between IGF-I and the IGF-I receptor and measures the net effects on IGF-I receptor activation. In a previous study we have shown that IGF-I bioactivity is positively related to total and free IGF-I levels obtained by IGF-I immunoassays. Interestingly, correlations were relatively weak (0.52 for total IGF-I and 0.20 for free IGF-I respectively), suggesting that the IGF-I KIRA assay produces new information about IGF-I signaling (Brugts et al., 2008).

We reported earlier that higher levels of IGF-I receptor stimulating activity were associated with a higher prevalence and a higher incidence of dementia (de Bruijn et al., 2014). In light of the conflicting results of the experimental and human studies, we aimed to test the long-term robustness of the association between IGF-I receptor stimulating activity and dementia risk by extending the follow up period with another 4 years and investigate possible effect modification by *ApoE*, the major genetic driver of AD and dementia risk.

MATERIALS AND METHODS

Setting

This study was embedded within the prospective, population-based Rotterdam Study, designed to study risk factors and determinants of chronic diseases in the elderly population. The Rotterdam Study began in 1990, with an invitation to inhabitants of 55 years and older residing in Ommoord, a district of Rotterdam in the Netherlands. Of the 10,215 people invited, 7,983 agreed to participate in the examinations at baseline. Up until 2015, there have been five follow-up examinations. Details of the study are described elsewhere (Ikram et al., 2017). Because IGF-I receptor stimulating activity was measured in blood samples collected at the second follow-up examination, between 1997 and 1999, this visit was used as baseline for the current study. Of the 5,990 participants that were alive in 1997–1999, 4,797 persons participated in the second follow-up assessment. IGF-I receptor stimulating activity levels were measured in blood samples of 1,050 randomly selected participants due to financial constraints. Five participants were excluded because their blood samples could not be correctly matched and 14 participants were excluded because measurements did not pass prior defined assay acceptance criteria (inter-assay coefficient of variation <10%). Another 17 participants were excluded because dementia screening was incomplete. Eventually, 1,014 participants were included in the analyses. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR[1]) and into the WHO International Clinical Trials Registry Platform (ICTRP[2]) under shared catalog number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Assessment of IGF-I Receptor Stimulating Activity

IGF-I receptor stimulating activity levels were measured using an IGF-I KIRA (intra- and inter-assay coefficients of variation of 5.2 and 12.2%, respectively; cross-reactivity of 15% for IGF-II; Chen et al., 2003; Brugts et al., 2008). Details of the assessment are described previously (de Bruijn et al., 2014).

Assessment of Dementia

Participants were screened for dementia at baseline and follow-up examinations using a 3-step protocol (Ott et al., 1998). First, screening was performed using the Mini-Mental State Examination (MMSE; Folstein et al., 1975) and the Geriatric Mental Schedule (GMS) organic level (Copeland et al., 1976). People with a MMSE score lower than 26 or GMS organic level higher than 0 were subsequently subjected to further examination and informant interview including the Cambridge Examination for Mental Disorders in the Elderly (CAMDEX; Roth et al., 1986). When necessary, participants underwent further neuropsychological assessment. When information on neuro-imaging was available, it was used as an aid for decision-making. For all suspected cases of dementia, the diagnosis was made by a consensus panel, led by a neurologist. During follow-up the cohort was under continuous surveillance for dementia incidence through electronic linkage of the database of the Rotterdam Study with medical records from general practitioners and the regional institute for outpatient mental health care (de Bruijn et al., 2015). The applied criteria for the diagnosis of dementia and probable AD are in accordance with the standard criteria for dementia (Diagnostic and Statistical Manual of Mental Disorders III-revised; American Psychiatric Association, 1987) and AD (National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association; McKhann et al., 2011). The total cohort was continuously monitored for incidence of dementia through linkage to the digitized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care. Follow-up for incident dementia is complete until January 2015.

[1] www.trialregister.nl

[2] http://www.who.int/ictrp/network/primary/en/

Other Measurements

Information on *ApoE* genotype was obtained using polymerase chain reaction on coded DNA samples. ApoE-ε4 carrier status was defined as carrier of one or two ε4 alleles. Blood pressure was calculated as the average of two measurements at the right brachial artery using a random-zero sphygmomanometer. Hypertension was defined as a blood pressure ≥140/90 mmHg or use of blood pressure lowering medication, prescribed for the indication of hypertension. Waist circumference was measured in centimeters. Serum glucose, total cholesterol, and high-density lipoprotein (HDL)-cholesterol levels were acquired by an automated enzymatic procedure (Boehringer Mannheim System). Missing values in covariates (for ApoE-ε4 carrier status 4.8%, for all other covariates less than 3.5%) were imputed based on age and sex.

Statistical Analyses

We examined the association between IGF-I receptor stimulating activity and incident dementia using Cox proportional hazards models. IGF-I receptor stimulating activity was entered per standard deviation (SD) into the models. We also studied IGF-I receptor stimulating activity in tertiles, using the lowest tertile as reference. All models were adjusted for age and sex (Model I) and additionally for hypertension, glucose, waist circumference, ApoE-ε4 carrier status, total cholesterol, and HDL-cholesterol (Model II) for being potential confounders. To investigate possible effect modification by ApoE, the (multiplicative) interaction between ApoE-ε4 carrier status and IGF-I receptor stimulating activity on dementia risk was tested using interaction terms and separate analyses on data stratified on ApoE-ε4 carrier status were performed. The underlying time-scale in the Cox proportional hazards models was the follow-up time, which was defined from time at blood sample collection (1997–1999) until the end of December 2015. Participants were censored within this time period when they were diagnosed with dementia, died, or decided to terminate their participation in the study. We separately investigated the association between IGF-I receptor stimulating activity and AD. Analyses were performed using IBM SPSS statistics version 24.0 (IBM Corp, Armonk, NY, USA).

TABLE 1 | Baseline characteristics.

	Prevalent dementia *N* = 30	At risk for incident dementia *N* = 984
Age, years	81.54 (8.33)	72.04 (7.1)
Females	70%	55.8%
IGF-IRSA, pmol/L	208.13 (77.59)	179.06 (55.48)
Apolipoprotein E-ε4 carrier status	71.4%	27.4%
Hypertension	82.1%	75.5%
Waist circumference, cm	94.17 (8.33)	93.84 (11.1)
Glucose, mmol/L	6.03 (1.07)	6.01 (1.51)
Total cholesterol, mmol/L	5.52 (1.17)	5.83 (1)
HDL-cholesterol, mmol/L	1.34 (0.46)	1.38 (0.37)

Data are presented as means (standard deviations) or percentages. N, number of people; IGF-IRSA, insulin-like growth factor I receptor stimulating activity; HDL, high-density lipoprotein.

RESULTS

Baseline characteristics of the study population are provided in **Table 1**. At baseline, 31 participants suffered from prevalent dementia, of which 23 had AD. During a follow-up of 10,752 person-years (mean follow-up of 11 years, SD 5.2 years), 174 participants developed dementia, of whom 140 were diagnosed with AD.

In the overall proportional hazard analyses, there was no statistically significant evidence for a relation between the level of IGF-I receptor stimulating activity at baseline and risk of dementia. However, the hazard ratio (HR) per SD increase in IGF-I receptor stimulating activity [1.11; 95% confidence interval (CI) 0.97–1.28; see **Table 2**] was very similar to the HR reported in our previous analyses (1.15; 95% CI 1.00–1.33) with shorter follow-up (de Bruijn et al., 2014). A congruent HR was found for the incidence of AD [HR 1.10 (95% CI 0.95–1.28)].

TABLE 2 | IGF-I receptor stimulating activity and risk of incident dementia.

	Dementia	Alzheimer's disease
	HR (95% CI)	HR (95% CI)
	n/N 174/973	n/N 140/973
Model I	1.09 (0.95–1.25)	1.07 (0.92–1.25)
Model II	1.11 (0.97–1.28)	1.10 (0.95–1.28)

Data are presented as hazard ratios (HR) and 95% confidence intervals (CI). N, number of people at risk for incident dementia; n, number of cases of incident dementia. IGF IRSA, insulin-like growth factor I receptor stimulating activity. Model I: adjusted for age and sex. Model II: adjusted for age, sex, hypertension, glucose, waist circumference, Apolipoprotein E-ε4 status, total cholesterol, and HDL cholesterol.

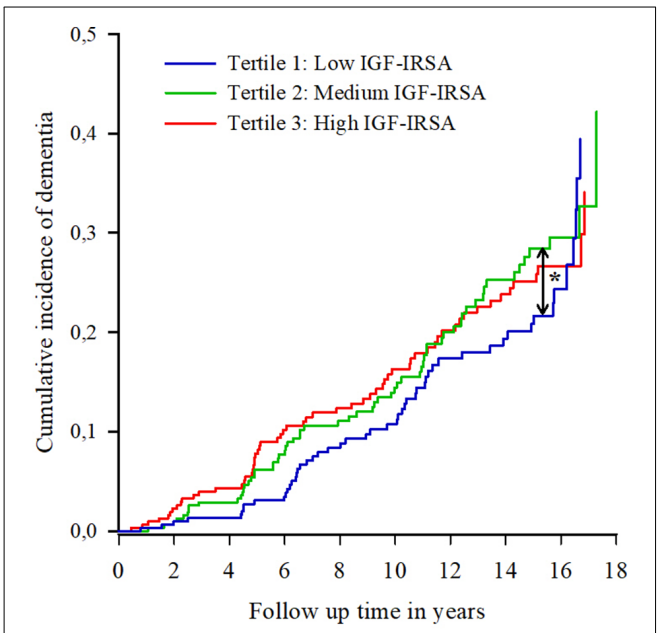

FIGURE 1 | Cumulative incidence curves of dementia per tertile of insulin-like growth factor (IGF-I) receptor stimulating activity (IGF-IRSA). Tertile 1 represents the lowest levels of IGF-I receptor stimulating activity, tertile 2 medium levels and tertile 3 the highest levels. *indicates significant at $p < 0.05$.

TABLE 3 | IGF-I receptor stimulating activity stratified by apolipoprotein E (ApoE) group.

	Non-carriers *N* = 680	ApoE-ε4 heterozygotes *N* = 240	ApoE-ε4 homozygotes *N* = 17	P for trend *N* = 937
IGF-IRSA (pmol/L)	181.92 (58.58)	174.31 (48.24)	151.82 (34.98)	F(2) 3.20, $p = 0.04$

Data are presented as means (standard deviations). N, number of persons; IGF-IRSA, insulin-like growth factor I receptor stimulating activity. Adjusted for age and sex.

TABLE 4 | IGF-I receptor stimulating activity tertile groups and risk of incident dementia.

	Dementia		Alzheimer's disease	
	ApoE–ε4+ n/N 65/255	ApoE–ε4– n/N 97/669	ApoE–ε4+ n/N 51/255	ApoE–ε4– n/N 78/669
IGF-IRSA groups	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Medium	3.80 (1.90–7.60)	0.97 (0.59–1.60)	3.44 (1.61–7.34)	1.00 (0.57–1.74)
High	2.71 (1.37–5.38)	1.09 (0.67–1.77)	2.38 (1.12–5.08)	1.01 (0.86–1.75)
Medium vs. High	1.40 (0.79–2.48)	0.89 (0.54–1.46)	1.44 (0.75–2.76)	0.99 (0.57–1.73)

Data are presented as hazard ratios (HR) and 95% confidence intervals (CI). N, number of people at risk for incident dementia. n, number of cases of incident dementia. IGF-IRSA, insulin-like growth factor I receptor stimulating activity divided in tertile groups. The lowest tertile group is used as reference. Adjusted for age, sex, hypertension, glucose, waist circumference, total cholesterol, and HDL cholesterol.

Figure 1 shows the cumulative incidence curves of dementia per tertile of IGF-I receptor stimulating activity. Proportional hazard analyses of dementia incidence revealed that those in the lowest tertile of IGF-I receptor stimulating activity at baseline had the lowest risk of dementia [HR moderate vs. low: 1.45 (95% CI 1.00–2.12); HR high vs. low 1.40 (95% CI 0.96–2.04)], while there was no difference in risk of dementia between the medium and highest tertiles (**Figure 1**).

IGF-I Receptor Activity and ApoE-ε4

At baseline there was a statistically significant difference in IGF-I receptor stimulating activity between ApoE-ε4 genotype groups without dementia (non-carrier, heterozygote and homozygote), after adjustment for age and sex ($p = 0.04$; see **Table 3**). The levels of IGF-I receptor stimulating activity were significantly lower in homozygotes for ApoE-ε4 than in people with no copies of the ApoE-ε4 allele ($p = 0.04$). There were no statistically significant differences in level of IGF-I receptor stimulating activity between non-carriers and ApoE-ε4 heterozygotes or ApoE-ε4 heterozygotes and homozygotes.

When testing for effect modification, significant evidence for a multiplicative interaction between IGF-I receptor stimulating activity and ApoE-ε4 carrier status was observed ($\chi^2(2) = 10.85$, $p = 0.004$). In those without the ApoE-ε4 variant, the level of IGF-I receptor stimulating activity was not associated with the risk of dementia (medium vs. low: HR 0.97 (95% CI 0.59–1.60); high vs. low: HR 1.09 (95% CI 0.67–1.77); $\chi^2(2) = 0.24$, $p = 0.89$). For those with one or more copies of the ApoE-ε4 allele, level of IGF-I receptor stimulating activity was positively associated with dementia risk. Dementia risk was significantly increased in people with one or more copies of the ApoE-ε4 allele and IGF-1 receptor stimulating activity in the median and top tertiles compared to those with IGF-I receptor stimulating activity in the bottom tertile [medium vs. low: HR 3.80 (95% CI 1.90–7.60); high vs. low: HR 2.71 (95% CI 1.37–5.38)].

Similar results were found for the incidence of AD (**Table 4**). **Figures 2A,B** show the cumulative incidence of dementia per tertile group of IGF-I receptor stimulating activity, stratified by ApoE-ε4 genotype.

DISCUSSION

In the extended follow-up period of 16 years, our study did not find evidence for a long-term dose response association between circulating IGF-I receptor stimulating activity at baseline and the future risk of dementia. Interestingly, we found evidence of an interaction between ApoE-ε4 and IGF-I receptor stimulating activity. In those hetero- and homozygous for the ApoE-ε4 allele, dementia risk was increased in persons with medium and high levels of IGF-I receptor stimulating activity at baseline, compared to those with low IGF-I receptor stimulating activity at baseline. However, no relation between IGF-I receptor stimulating activity and dementia risk was observed in non-carriers of the ApoE-ε4 allele. This suggests that, in ApoE-ε4 carriers, there is a certain threshold above which IGF-I receptor stimulating activity becomes associated with dementia at long-term follow up. In addition, in individuals without dementia, IGF-I receptor stimulating activity was lower in homozygote carriers of ApoE-ε4 than in people with other ApoE genotypes.

To our knowledge the Rotterdam Study is still the only study that has investigated the role of circulating IGF-I receptor stimulating activity in relation to dementia. As circulating IGF-I receptor stimulating activity is only modestly correlated to total serum IGF-I and IGF-I/IGFBP-3 ratio (Brugts et al., 2008), comparison to other studies on serum total IGF-I, measured by immunoassays, and dementia risk, described by Ostrowski et al. (2016) is difficult.

We found a modifying effect of ApoE-ε4 on circulating IGF-I receptor stimulating activity at baseline and an interaction on the relation between IGF-I receptor stimulating activity and the risk of dementia. The observed interaction between circulating IGF-I receptor stimulating activity and ApoE isoforms in our study could reflect opposing influences on shared pathways

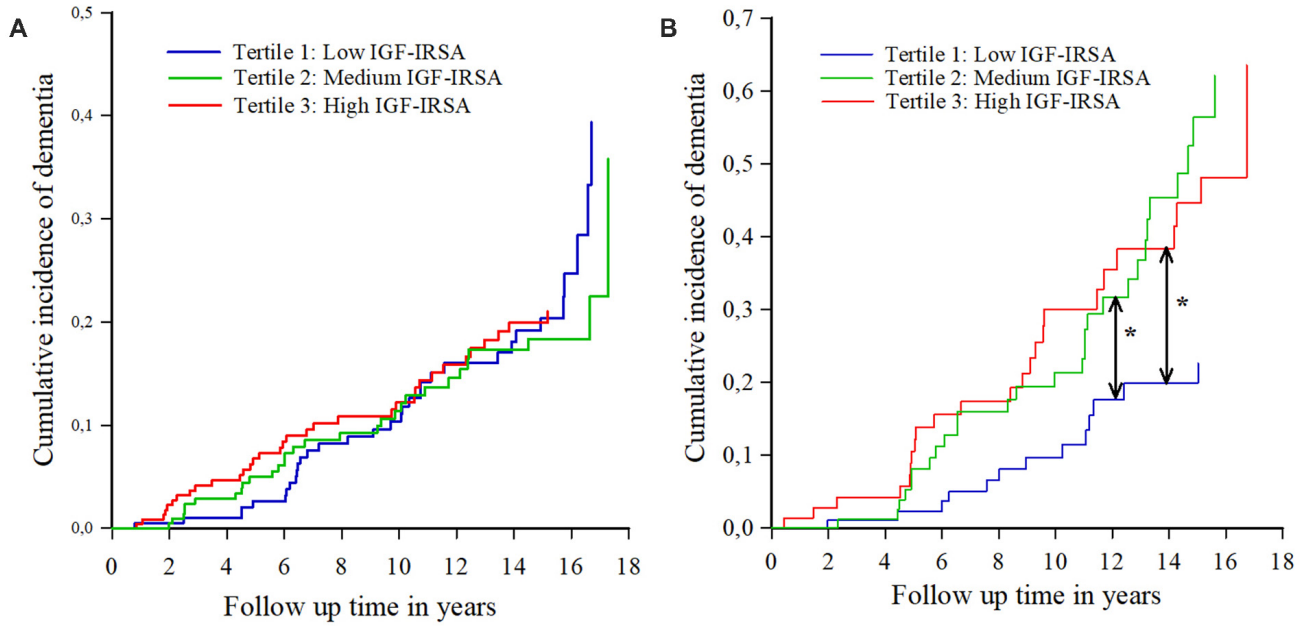

FIGURE 2 | (A) Cumulative incidence curves of dementia per tertile of IGF-I receptor stimulating activity (IGF-IRSA), for persons without apolipoprotein E ε4 allele (ApoE-ε4). Tertile 1 represents the lowest levels of IGF-I receptor stimulating activity, tertile 2 medium levels and tertile 3 the highest levels. **(B)** Cumulative incidence curves of dementia per tertile of IGF-I receptor stimulating activity (IGF-IRSA), for hetero- and homozygotes of the ApoE-ε4 allele. Tertile 1 represents the lowest levels of IGF-I receptor stimulating activity, tertile 2 medium levels and tertile 3 the highest levels. *Indicates significant at $p < 0.05$.

involved in Alzheimer pathology. Both ApoE and IGF-I are involved in the regulation of AD biomarkers: IGF-I is an important mediator in the clearance and regulation of Aβ in the brain, enhances survival of neurons exposed to Aβ and inhibits tau phosphorylation (Doré et al., 1997; Carro et al., 2002, 2006; Cheng et al., 2005; Engel et al., 2006; Moloney et al., 2010; Talbot et al., 2012). The ApoE-ε4 allele, on the other hand, is associated with decreased $A\beta_{1-42}$ and higher tau and p-tau in the CSF and increased cerebral amyloid deposition across the AD spectrum (Tapiola et al., 2000; Morris et al., 2010; Leoni, 2011; Risacher and Saykin, 2013; Wildsmith et al., 2013; Kanekiyo et al., 2014). In mice expressing human ApoE-ε4, increased tau phosphorylation has been demonstrated (Tesseur et al., 2000). ApoE-ε4 and IGF-I also have an opposing influence on NMDA receptor signaling. The NR2B subunit of the NMDA receptor, in particular, is suggested to be of specific importance for spatial learning and long-term potentiation, impaired in AD (Sonntag et al., 2000; Le Grevès et al., 2005; Reiman et al., 2009). The ApoE-ε4 genotype is associated with decreased NR2B NMDA receptor subunit levels and enhances age-related decline in cognitive function by down-regulating signaling in mice (Liu et al., 2015). In contrast, IGF-I positively affects the NMDA receptor pathway by increasing the NR2B subunit mRNA transcript of the hippocampal NMDA receptor in rats (Sonntag et al., 2000; Le Grevès et al., 2005). The observed association between elevated levels of IGF-I receptor stimulating activity and increased risk of dementia in ApoE-ε4 carriers might thus be a reflection of a compensatory response to neuropathological changes associated with the ApoE-ε4 genotype and a preclinical loss of sensitivity of the IGF-I receptor.

The strengths of our study are its prospective, population-based design, the long follow-up period, and the use of a direct measure of circulating IGF-I receptor stimulating activity. However, there are also some limitations. First, IGF-I receptor stimulating activity was only measured in peripheral blood samples. Even though circulating IGF-I crosses the blood-brain barrier, we could not assess the extent to which our measurements of circulating IGF-I receptor stimulating activity are related to actual IGF-I receptor stimulating activity in the brain (Reinhardt and Bondy, 1994). In addition, IGF-I has important autocrine and paracrine actions at the tissue level. However, IGF-I measured by the KIRA assay may not necessarily reflect IGF-I bioactivity at the local tissue level (Chen et al., 2003). Second, no total serum IGF-I levels were measured in our study, therefore we were unable to compare the relationship of IGF-I receptor stimulating activity and total IGF-I with dementia and to show that measuring IGF-I bioactivity with the IGF-I KIRA assay provides other insights about the role of IGF-I in dementia than the measurement of total IGF-I. Third, IGF-I receptor stimulating activity was assessed at the second follow-up visit of the Rotterdam Study, which might have led to survival effects in the study population which was included at baseline.

In conclusion, our current study sheds new light on the association between IGF-I signaling and the neuropathology of dementia, suggesting a threshold effect of IGF-I receptor stimulating activity moderated by ApoE genotype, since only

for those with one or more copies of the ApoE-ε4 allele and in the lowest tertile of IGF-I receptor stimulating activity the risk of future dementia is decreased. Our study suggests that the ApoE-ε4 genotype modifies the relationship between IGF-I receptor stimulating activity and dementia and elevated IGF-I receptor stimulating activity levels mark a compensatory response to neuropathological changes associated with the ApoE-ε4 genotype. In line with the hypothesis that low IGF-I activity increases the risk of dementia, we found the ApoE-ε4 homozygotes, with a lifetime risk of AD of 80% (van der Lee et al., 2018), have the lowest IGF-I levels. This provides a genetic benchmark for the hypothesis that low IGF-I receptor stimulating activity is associated with an increased risk of AD. However, our findings require replication in other cohorts, reusing measures of IGF-I receptor stimulating activity rather than total IGF-I serum levels as putative predictors of dementia risk.

AUTHOR CONTRIBUTIONS

CD, MI, ES and SG contributed to the study concept and design. JJ and MB were responsible for the collection and assessment of IGF-I kinase receptor activation assay. SG and AS performed the statistical analyses. CD, JJ, MD and SG contributed to the interpretation of the results. SG drafted and revised the manuscript. All authors contributed to the critical revision of the manuscript. All authors approve the final manuscript as submitted and agree to be accountable for all aspects of the work.

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[3]www.costream.eu

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Hapln2 in Neurological Diseases and Its Potential as Therapeutic Target

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Hyaluronan and proteoglycan link protein 2 (Hapln2) is important for the binding of chondroitin sulfate proteoglycans to hyaluronan. Hapln2 deficiency leads to the abnormal expression of extracellular matrix (ECM) proteins and dysfunctional neuronal conductivity, demonstrating the vital role of Hapln2 in these processes. Studies have revealed that Hapln2 promotes the aggregation of α-synuclein, thereby contributing to neurodegeneration in Parkinson's disease (PD), and it was recently suggested to be in intracellular neurofibrillary tangles (NFTs). Additionally, the expression levels of Hapln2 showed lower in the anterior temporal lobes of individuals with schizophrenia than those of healthy subjects. Together, these studies implicate the involvement of Hapln2 in the pathological processes of neurological diseases. A better understanding of the function of Hapln2 in the central nervous system (CNS) will provide new insights into the molecular mechanisms of these diseases and help to establish promising therapeutic strategies. Herein, we review the recent progress in defining the role of Hapln2 in brain physiology and pathology.

Keywords: Hapln2, aggregates, Parkinson's disease, Alzheimer's disease, schizophrenia

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INTRODUCTION

Parkinson's disease (PD), Alzheimer's disease (AD) and schizophrenia are neurological diseases characterized by the dysfunction of certain types of neurons (Hardy and Higgins, 1992; Korth, 2012; Ghosh et al., 2016). However, the molecular mechanisms underlying the pathological processes of these brain disorders remain elusive. Accumulating evidence suggests that the pathogenesis of these diseases involve abnormal protein aggregates (Hardy and Higgins, 1992; Korth, 2012; Ghosh et al., 2016). For example, in PD, the formation of Lewy bodies comprising α-synuclein aggregates leads to the degeneration of dopaminergic neurons in the substantia nigra (SN; Ghosh et al., 2016; Wang et al., 2016). The deposition of amyloid beta protein induces neuronal cell death in the development of AD (Hardy and Higgins, 1992). Recently, schizophrenia has been linked with the abnormal deposition of disrupted in schizophrenia 1 (DISC1) aggregates (Atkin and Kittler, 2012; Korth, 2012). Moreover, all of these three diseases have been linked with the dysfunction of the ubiquitin-proteasome pathway (UPP; Lam et al., 2000; Bousman et al., 2010; Shen et al., 2013), which balances protein synthesis with degradation (Tsukamoto and Yokosawa, 2006). Substrates for degradation within this pathway are specified by E3 ubiquitin ligases (Ardley and Robinson, 2005), and we recently demonstrated that the E3 ubiquitin ligases Hrd1, Gp78 and Parkin colocalized with hyaluronan and proteoglycan link protein 2 (Hapln2; Wang et al., 2016).

Studies have shown that Hapln2, also known as brain-derived link protein1 (Bral1), is vital for neuronal conductivity and the formation of the extracellular matrix (ECM), and besides its potential role in the UPP, it has been identified as a contributor to the pathological processes in several neurological disorders. For example, Martins-de-Souza et al. (2009) showed that the expression levels of Hapln2 in the anterior temporal lobe are lower in patients with schizophrenia than those of the control subjects, and Minjarez et al. (2013) suggested that Hapln2 was probably in the neurofibrillary tangles (NFTs) from AD brain. Our studies recently revealed that Hapln2 expression levels were dramatically increased in the SN of PD patients and in the 6-hydroxydopamine-induced rat PD model (Liu et al., 2015; Wang et al., 2016). This review article summarizes recent progress focusing on the roles of Hapln2 in the central nervous system (CNS) under physiological and pathological conditions, highlighting the therapeutic potential of Hapln2 in neurological diseases.

HAPLN2 IN CNS PHYSIOLOGY

Hapln2 Protein Structure

The structure and roles of Hapln2 in the CNS have been studied since around the turn of this century, when an analysis of the gene now known as *HAPLN2* revealed seven exons encoding a polypeptide with an estimated molecular mass of 38 kDa (Hirakawa et al., 2000). The Hapln2 protein comprises three modules, namely, a proteoglycan tandem repeat 1 (PTR1) domain, a PTR2 domain, and an immunoglobulin-like fold (Hirakawa et al., 2000; Spicer et al., 2003). Western blotting results from Hapln2-EGFP transfected cell lysates have shown that an anti-Hapln2 antibody recognizes a band of around 55 kDa, which is in accordance with the predicted molecular mass (Wang et al., 2016). However, a band for Hapln2 of ∼48 kDa is detected from SN tissue lysates of adult rats, revealing an inconsistency between the lysates from transfected cells and those from adult brain tissues (Wang et al., 2016). Given that Hapln2 was a kind of link protein, we attributed this discrepancy to hyaluronic acid modifications.

Hapln2 Expression

The hyaluronan and proteoglycan link family of proteins consists of four members: Hapln1, Hapln2, Hapln3, and Hapln4 (Spicer et al., 2003). An amino acid sequence alignment demonstrated that these four proteins have similarities of ∼52%–62% (Spicer et al., 2003), with a high level of conservation among vertebrate species. Northern analyses and EST database searches revealed that Hapln2 and Hapln4 are specifically expressed in the brain, with the levels of Hapln2 significantly higher than that of Hapln4 (Spicer et al., 2003). These data indicate that the four-link proteins may have different roles.

Northern blot analyses by Hirakawa et al. (2000) showed that *HAPLN2* mRNA is expressed at high levels in the human hippocampus, medulla oblongata, putamen, SN, thalamus, and spinal cord but at lower levels in the cerebellum, cerebral cortex, frontal lobe, and nucleus accumbens. By *in situ* hybridization, we detected high expression levels of *Hapln2* mRNA in the SN, olfactory bulb, red nucleus, cerebellum, brain stem, and hippocampus but relatively weak signals in the cerebral cortex of adult rat brain (Wang et al., 2016). The differential expression of Hapln2 among the various brain regions suggests that the protein has different roles in areas of high expression (SN and hippocampus) than in areas of low expression (cerebral cortex). The discrepancy between these studies regarding the expression in the cerebellum may reflect species specificity and/or the different experimental approaches used.

Hapln2 in ECM Formation and Neuronal Conductivity

Lecticans such as brevican and aggrecan mainly bind to the hyaluronans, which are the major components of the ECM in the brain (Yamaguchi, 2000; Theocharis et al., 2010). The proposed function of the link proteins is to stabilize these binding interactions (Oohashi et al., 2002; Bekku et al., 2003). Northern blot analyses revealed that the expression of Hapln2 largely coincides with that of brevican, a brain-specific lectican (Hirakawa et al., 2000). Previous studies have shown that aggrecan aggregates are denser in the presence of link protein (Mörgelin et al., 1988, 1992; Oohashi et al., 2002). Moreover, immunohistochemical analyses showed that the levels of ECM proteins, such as versican V2, brevican, hyaluronan, and tenascin-R, are much lower in the brain of mice with Hapln2 deficiency (Bekku et al., 2010). These findings not only reveal the proximity of Hapln2 and lecticans in the brain but also indicate the potential role of Hapln2 in stabilizing the binding between brain lecticans and hyaluronan (Hirakawa et al., 2000; **Figure 1**).

Northern blot analyses have shown that Hapln2 expression in the mouse brain begins at 20 days after birth and increases with age (Hirakawa et al., 2000). Immunohistochemical analyses revealed that the patterns of Hapln2 and versican V2 expression are similar in the late developmental stage of cerebellar development and in the adult mouse brain (Oohashi et al., 2002). Moreover, Hapln2 colocalizes with versican at myelinated retinal ganglion cell axons (Oohashi et al., 2002). The Ranvier nodes could be marked by the voltage-gated Na^+ channels antibody (Oohashi et al., 2002). Interestingly, Hapln2 preferentially localizes with versican at nodes of Ranvier in adult mouse brain, as observed by labeling with antibodies against Hapln2, voltage-gated Na^+ channels and the contactin-associated protein (Rasband et al., 1999; Oohashi et al., 2002).

As Na^+ channel clustering coincides with myelination (Bekku et al., 2010), it was suggested that Hapln2 may also affect the clustering of the Na^+ channels. Of note, the clustering of Na^+ and K^+ channels is critical for saltatory conduction (Oohashi et al., 2002; Bekku et al., 2010). Although immunohistochemical analyses showed that Hapln2 deletion did not affect the clustering of Na^+ and K^+ channels, flash visual evoked potentials had a longer latency and smaller amplitude in recordings from Hapln2 knockout mice compared with those from wild-type mice (Bekku et al., 2010). Besides, consistent with the previous results mentioned above, immunostaining analysis demonstrated that there were no signals of ECM proteins including brevican, neurocan and versican at the CNS nodes in adult mice with

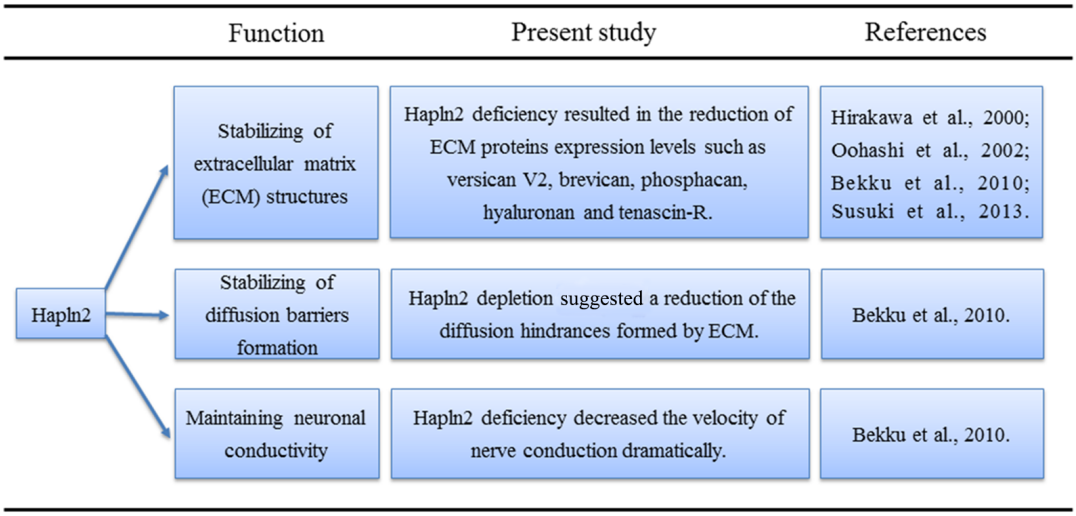

FIGURE 1 | Physiological functions of hyaluronan and proteoglycan link protein 2 (Hapln2) in the central nervous system (CNS).

Hapln2 deletion (Susuki et al., 2013), suggesting the essential role of Hapln2 in the formation of nodal ECM. Additionally, Susuki et al. (2013) showed that single disruption of ECM or paranodal barriers or axonal cytoskeletal scaffolds (CS) had mild effects on the development of nodes, which was consistent with the previous results to some degree (Bekku et al., 2010). However, disruption of the paranodal barriers and ECM, or paranodal barriers and CS, or ECM and CS resulted in juvenile lethality and the robust decrease of clustering of Na^+ channels (Susuki et al., 2013), which indicates the complex and complementary roles of the three elements in the formation of nodes. Early studies showed that clustering of nodal proteins such as neurofascin-186 (NF186) was responsible for action potential (AP) propagation (Salzer, 2003; Susuki et al., 2013). Further experiments suggested that there was a specific interaction between Hapln2 and NF186 using pull-down method (Susuki et al., 2013), suggesting the important role of Hapln2 in AP propagation. All of these data indicated the important roles of Hapln2 in clustering of Na^+ channel, nodes formation and AP propagation. However, more studies should be performed to clarify the details about the relationship between Hapln2 and the paranodal barriers or the CS in these processes during the development.

As mentioned above, Hapln2 deficiency decreased the expression of ECM-associated proteins (Bekku et al., 2010). Moreover, diffusion-weighted magnetic resonance imaging and real-time iontophoretic assays showed an increase in the diffusion signals from the ECM in white matter in animals with Hapln2 depletion (Bekku et al., 2010). What was more, Hapln2 was essential for nodal ECM formation and interacted with nodal protein NF186 directly (Susuki et al., 2013). Altogether, these results suggest that Hapln2 alters neuronal conductivity by affecting the extracellular diffusion barriers at the nodes of Ranvier rather than by directly impacting saltatory conduction (**Figure 1**).

HAPLN2 IN CNS PATHOLOGY

Hapln2 in PD

PD is characterized by the formation of Lewy bodies and selective loss of dopamine neurons in the SN (Liu et al., 2015; Wang et al., 2016). Despite the identification of a variety of risk factors including genetic elements, impairments in mitochondrial function and inflammatory responses, the pathogenesis of PD still remains unclear (Shao et al., 2013; Cruces-Sande et al., 2017; Billingsley et al., 2018; Segura-Aguilar and Huenchuguala, 2018). However, clinical analyses and animal experiments implicate the involvement of Hapln2 in PD. In a quantitative proteomics study, we demonstrated that the protein levels of Hapln2 were increased with the highest fold among all the upregulated proteins in the SN region of PD patients compared with the control subjects (Liu et al., 2015). This increase in the SN was confirmed both at the mRNA and protein levels by quantitative PCR and Western blotting analyses in a rat model of PD 2 weeks after 6-hydroxydopamine treatment as well as 4 weeks postlesion (Wang et al., 2016).

Flow cytometry and immunostaining analyses showed that the overexpression of Hapln2 increased the death of MES23.5 and primary neuronal cells (Wang et al., 2016). Confocal imaging of these cells demonstrated that the overexpressed Hapln2 formed aggregates, which were found to colocalize with E3 ubiquitin ligases Hrd1, Gp78, and Parkin (Wang et al., 2016). It is known that E3 ubiquitin ligases involve in specifying and catalyzing the transfer of substrates in the UPP (Uchida and Kitagawa, 2016). Moreover, the treatment of MES23.5 cells with the proteasome inhibitor MG132 increased Hapln2 levels, indicating that the UPP pathway regulates Hapln2 degradation (Wang et al., 2016). Of note, previous research showed that UPP dysfunction *via* proteasome inhibition resulted in the degeneration of dopaminergic neurons *in vitro* and *in vivo* (Kikuchi et al., 2003;

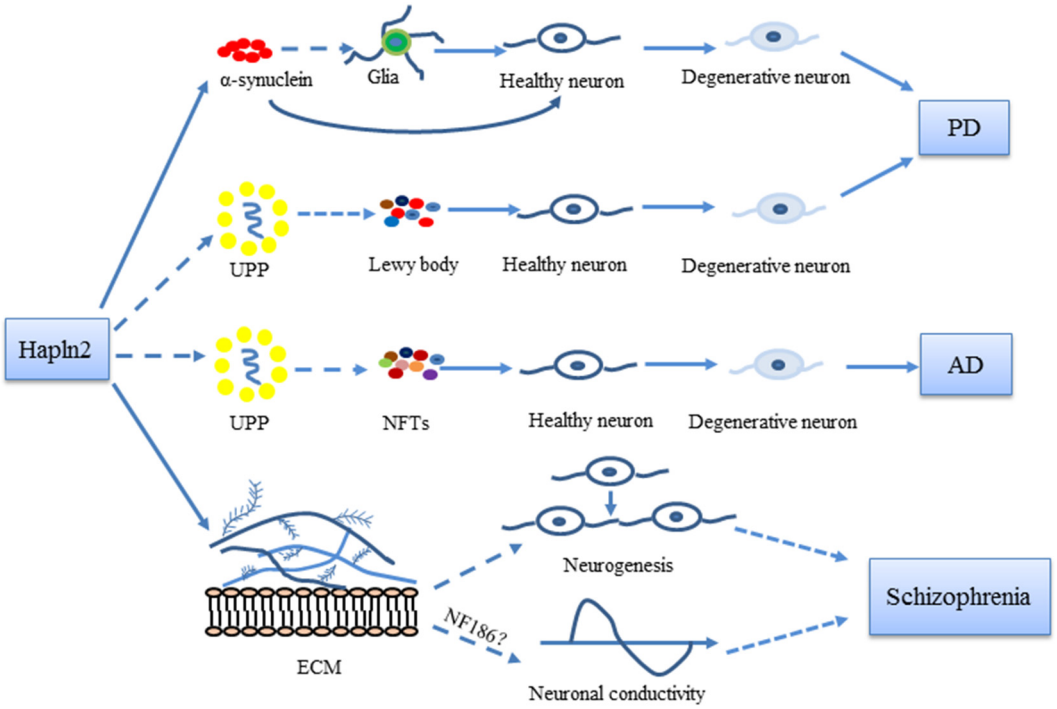

FIGURE 2 | Possible roles of Hapln2 in Parkinson's disease (PD), Alzheimer's disease (AD), and schizophrenia. An upregulation of Hapln2 is observed in the substantia nigra (SN) of PD brain. The high expression levels of Hapln2 promote the aggregation of α-synuclein, which contributes to the degeneration of dopamine neurons and the pathophysiology of PD. Hapln2 has also been suggested in neurofibrillary tangles (NFTs). There is evidence showing the vital role of extracellular matrix (ECM) proteins during neurogenesis and neuronal conductivity, which become dysfunctional in schizophrenia. As Hapln2 helps maintain the ECM, it may also play a role in schizophrenia. Hapln2 colocalizes with several E3 ligases of the ubiquitin-proteasome pathway (UPP). The influence of Hapln2 on the ECM and UPP may contribute to PD, AD, and schizophrenia, neurological diseases in which ECM and UPP dysfunction have been observed.

Sun et al., 2006). These and the more recent results suggest that the increase in Hapln2 as a result of UPP dysfunction contributes to PD pathology.

Immunostaining analysis of MES23.5 cells overexpressing Hapln2 also showed an accumulation of α-synuclein, aggregates of which were found to colocalize with Hapln2 (Wang et al., 2016). Thus, Hapln2 may be another component of Lewy bodies in PD, which primarily comprise α-synuclein (Castro-Sánchez et al., 2018). The accumulation of α-synuclein not only promotes the death of dopaminergic neurons but also activates glial cells (La Vitola et al., 2018; Martinez et al., 2018). Mice overexpressing wild-type α-synuclein exhibit marked microglial activation (Su et al., 2008). Pathological α-synuclein aggregates activate astrocytes (Yu et al., 2018). Moreover, knockout of Hapln2 significantly reduces the fraction of α-synuclein that is insoluble in extracts from SN and cerebellum of 6-month-old mouse brain (Wang et al., 2016). These findings suggest that Hapln2 may contribute to neurodegeneration by regulating the activation of glial cells in the brain (**Figure 2**).

Hapln2 in AD

A variety of environmental and genetic factors have also been implicated in the pathology of AD (Chin-Chan et al., 2015; Kikis, 2017; Kocahan and Doğan, 2017). However, due to the complexity of this disease, the underlying molecular mechanism remains largely unknown. Although a major hallmark of AD is the accumulation of intracellular NFTs (Manczak et al., 2018), their insolubility impedes the identification of their integral components (Perry et al., 1991; Minjarez et al., 2013). Nevertheless, Benito and colleagues used different solubilization methods to identify lots of polypeptides, including Hapln2, in total homogenates of AD brain tissue containing NFTs by tandem mass spectrometry (Minjarez et al., 2013; Sugawara et al., 2018). However, there was no direct evidence for a colocalization of Hapln2 with tau, the main component of NFTs.

Notably, both AD and PD are age-associated neurodegenerative diseases with characteristic protein aggregates (Bridi and Hirth, 2018; Daniele et al., 2018; Theofilas et al., 2018). As mentioned above, the overexpression of Hapln2 promotes the aggregate formation and is involved with neuron death and the UPP in the pathology of PD (Wang et al., 2016). As Hapln2 was found to colocalize with some E3 ligases (Wang et al., 2016), it may also contribute to AD pathology *via* disruptions of the UPP. Postmortem tissue samples from the hippocampus, superior and middle temporal gyri, parahippocampal gyri, and the inferior parietal lobes of AD patients exhibit signs of reduced proteasome activity compared with those from control subjects (Keller et al., 2000). However, further animal experiments and clinical studies are needed to determine whether Hapln2 is a major constituent of the aggregates in these diseases.

Although high levels of Hapln2 in the hippocampus were measured by *in situ* hybridization (Wang et al., 2016), the function of Hapln2 in this brain region remains unknown. The hippocampus is involved in learning and memory (Portero-Tresserra et al., 2018; Wang D. et al., 2018), the dysfunction of which is a major symptom of AD (Axelrud et al., 2018). Behavioral tests in a mouse model of AD revealed a significant impairment in contextual conditioning and performance in pattern separation tests after neurogenesis in the hippocampus was inhibited (Hollands et al., 2017). Moreover, the performance of AD transgenic mice in spatial learning and memory tasks improved when hippocampal neurogenesis was enhanced by deep brain magnetic stimulation (Zhen et al., 2017). Of note, the fate determination of neural stem cells is guided by ECM components, such as the heparan sulfate proteoglycans glypican and perlecan (Yu et al., 2017). Given the vital role of Hapln2 in maintaining the ECM scaffold (Bekku et al., 2010), whether Hapln2 is involved in AD pathology by regulating hippocampal neurogenesis needs further investigation.

Hapln2 in Schizophrenia

Although the pathophysiology of schizophrenia has not been fully defined, genetic factors, epigenetic elements, and abnormal neurotransmission in the hippocampus are important contributors (Kim et al., 2018; Sugawara et al., 2018; Wang H. Y. et al., 2018). Previous studies of postmortem brain tissues from patients with schizophrenia revealed the presence of insoluble aggregates containing DISC1 (Atkin and Kittler, 2012; Korth, 2012). The overexpression of DISC in neuroblastoma cells leads to the formation of aggresomal deposits (Korth, 2012), which can invade cells and recruit other proteins that may contribute to the impairment of neuronal cells (Atkin and Kittler, 2012; Korth, 2012). Hapln2 represents another potential contributor, as a shotgun proteomic analysis of postmortem anterior temporal lobe tissues showed that Hapln2 protein levels are lower in schizophrenia patients than in control subjects (Martins-de-Souza et al., 2009). Additionally, a convergent pathway analysis indicated that there is dysregulation of the UPP in schizophrenia (Bousman et al., 2010). As mentioned above, the UPP is vital for protein catabolism *via* the degradation of particular substrate proteins (Tsukamoto and Yokosawa, 2006). The colocalization of Hapln2 with E3 ligases revealed in our previous work suggests a potential function of Hapln2 in the UPP (Wang et al., 2016), which suggesting that Hapln2 may also contribute to the pathophysiology of schizophrenia through dysfunction of UPP.

Moreover, it has been reported that ECM plays important roles in regulating neurogenesis, axonal outgrowth, synaptogenesis and cell migration (Bandtlow and Zimmermann, 2000; Faissner et al., 2010; Gundelfinger et al., 2010). The analysis of the schizophrenia risk genes showed that most of the genes were involved in the regulation of neuronal migration and cell adhesion (O'Dushlaine et al., 2011; Lips et al., 2012; Aberg et al., 2013). Additionally, the disruption of conduction velocity has been suggested in patients of schizophrenia (Thaker, 2008; Takahashi et al., 2011). The study of schizophrenia animal models indicated the reduction of conduction velocity, indicating the important role of nerve conduction in schizophrenia (Roy et al., 2007; Tanaka et al., 2009; Takahashi et al., 2011). As one of the important components of ECM, Hapln2 may be involved in the pathogenesis of schizophrenia through regulating the neuronal migration and velocity of nerve conduction. More studies should focus on the molecular mechanism of Hapln2 in the pathogenesis of schizophrenia using animal models and clinical analysis.

CONCLUSIONS

As a brain specific-hyaluronan and proteoglycan link protein, Hapln2 plays vital physiological roles in the formation and maintenance of the ECM scaffold. Studies in knockout mice have also revealed that Hapln2 influences neuronal conductivity. Several studies have revealed the involvement of Hapln2 in the pathogenesis of neurological diseases including PD, AD and schizophrenia, which provided new insights into the underlying molecular mechanisms of brain disorders (**Figure 2**).

Current research suggested that Hapln2 was involved in the formation of α-synuclein aggregates in PD pathology and study in AD suggested that Hapln2 might be the component of NFTs aggregates (Minjarez et al., 2013; Wang et al., 2016). It is noteworthy that, besides PD, AD and Schizophrenia, dysfunction of UPP system has also been implicated in some other neurological diseases including Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS; Zhao et al., 2016; Desai et al., 2018; Harding and Tong, 2018; Thibaudeau et al., 2018). Thus, it is likely that Hapln2 may also be involved in the pathological processes of these diseases through the UPP system. However, further studies of animal and clinical experiments are needed to clarify these mechanisms and the precise role that Hapln2 plays in these neurological disorders in the future. Nevertheless, the restriction of Hapln2 expression to the brain suggests that it is an important contributor.

AUTHOR CONTRIBUTIONS

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REVIEW
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Insulin Signaling Impairment in the Brain as a Risk Factor in Alzheimer's Disease

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Type 2 diabetes is a risk factor for developing Alzheimer's disease (AD). The underlying mechanism that links up the two conditions seems to be the de-sensitization of insulin signaling. In patients with AD, insulin signaling was found to be de-sensitized in the brain, even if they did not have diabetes. Insulin is an important growth factor that regulates cell growth, energy utilization, mitochondrial function and replacement, autophagy, oxidative stress management, synaptic plasticity, and cognitive function. Insulin desensitization, therefore, can enhance the risk of developing neurological disorders in later life. Other risk factors, such as high blood pressure or brain injury, also enhance the likelihood of developing AD. All these risk factors have one thing in common – they induce a chronic inflammation response in the brain. Pro-inflammatory cytokines block growth factor signaling and enhance oxidative stress. The underlying molecular processes for this are described in the review. Treatments to re-sensitize insulin signaling in the brain are also described, such as nasal insulin tests in AD patients, or treatments with re-sensitizing hormones, such as leptin, ghrelin, glucagon-like peptide 1 (GLP-1),and glucose-dependent insulinotropic polypeptide (GIP). The first clinical trials show promising results and are a proof of concept that utilizing such treatments is valid.

Keywords: growth factor, brain, GLP-1, mitochondria, apoptosis, autophagy, inflammation

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INTRODUCTION

Recently, type 2 diabetes mellitus (T2DM) has been identified as a risk factor for Alzheimer's disease (AD). Epidemiological studies of patient data sets have found a clear correlation between T2DM and the risk of developing AD or other neurodegenerative disorders (Hoyer, 1998; Luchsinger et al., 2004; Ristow, 2004; Strachan, 2005; Haan, 2006). In one study, 85% of AD patients had diabetes or showed increased fasting glucose levels, compared to 42% in age-matched controls (Janson et al., 2004). In longitudinal studies of cohorts of people, it was found that glucose intolerance was a good predictor for the development of dementia later in life (Schrijvers et al., 2010; Ohara et al., 2011; Li T.C. et al., 2017). In the Hisayama study, a total of 1,017 dementia-free subjects aged ≥60 years were tested for glucose tolerance and followed up for 15 years. It was found that the glucose intolerance correlated well with the development of vascular dementia and AD in later life (Ohara et al., 2011).

When analyzing the brain tissue of AD patients, it was observed that insulin signaling was much desensitized, even in AD patients that did not have T2DM (Frolich et al., 1998). One study found that the levels of insulin, IGF-1 and IGF-II were much reduced in brain tissue. In addition, levels of the insulin receptor, the insulin-receptor associated PI3-kinase, and activated Akt/PKB kinase

were much reduced (Steen et al., 2005). A second study found increased levels of IGF-1 receptors and the localization of insulin receptors within cells rather than on the cell surface where they could function. Decreased levels of insulin receptor substrates IRS-1 and IRS-2 levels were observed in neurons, and increased levels of inactivated phospho (Ser^{312})IRS-1 and phospho (Ser^{616})IRS-1 (Moloney et al., 2010). A third study analyzed the biochemical changes in the brains of AD patients in great detail, and also analyzed the functionality of insulin signaling using an *ex vivo* insulin incubation technique. In this study, tissue from the hippocampal formation was incubated with insulin to measure any biochemical changes. It was found that the second messenger cascade activated by insulin was significantly impaired. For example, the activation of the downstream kinase Akt/PKB at the $Serine^{473}$ site was almost completely abolished, while the activation was effective in age-matched control brain tissue (Talbot et al., 2012).

INSULIN IS A KEY GROWTH FACTOR

Insulin is not just a hormone that regulates blood glucose levels, but it is also an important growth factor that regulates neuronal growth, repair, and functions. The insulin receptor (IR) activates gene expression via MAPkinase and Akt/PKB cell signaling that enhances glucose uptake, mitochondrial function and replacement, protein synthesis, autophagy, and the inhibition of apoptosis (Carro and Torres-Aleman, 2004; Schubert et al., 2004; Heras-Sandoval et al., 2014; Holscher, 2014; Najem et al., 2014; see **Figure 1**). Among other key physiological functions, it also activates the Nrf2 promotor to activate gene expression that protects cells against oxidative stress (Song et al., 2018). Synaptic activity in the brain and the integrity of neuronal networks is also regulated by insulin (Ferrario and Reagan, 2018). It is, therefore, plain to see how the reduction of insulin signaling in the brain impairs cell maintenance and repair and puts neurons at risk to develop neurodegenerative disorders over time. As neurons in the brain are not replaced, the damage will accumulate and may present itself as AD in old age.

CAUSES FOR INSULIN DESENSITIZATION

As there are few genetic links to AD, the disease is predominantly a "sporadic"condition that is triggered by environmental influences. Only around 1–5% of cases can be linked to a clear genetic origin, such as a mutation in the amyloid precursor protein (APP) or presenilin-1 gene (Blennow et al., 2006; Guerreiro and Hardy, 2014; Karch and Goate, 2015). The key question is: what else causes AD and what are the drivers of disease progression?

Chronic Inflammation

Under physiological conditions, there is no inflammation response in the brain. However, chronic exposure to inflammatory triggers can induce a chronic inflammation response in the brain that is detrimental for neuronal health and survival. Chronic neurodegenerative disorders are always accompanied by a robust chronic inflammation response, and there is an agreement that this response is a key driver for neurodegenerative disorders (Akiyama et al., 2000; Perry et al., 2007; Lee et al., 2010; Clark and Vissel, 2018). The inflammation response activates microglia in the brain and enhances the release of oxidative stressors, such as nitric oxide and pro-inflammatory cytokines which inhibit growth factor signaling (Cunningham et al., 2005; Vaz et al., 2011). It is of interest to note that key risk genes for AD are linked to the control and progression of the inflammation response. The APOEe4 allele is a major risk factor for developing AD (Blacker et al., 1997). APOE is a pro-inflammatory cytokine that is released by activated microglia (Krasemann et al., 2017). APOE can inhibit receptors of the reelin family that activate intracellular signaling similar to the IR and that modulate glucose uptake, energy utilization, cell growth, and synaptic plasticity, as well as reduce the inflammation response (Herz and Chen, 2006; Lane-Donovan et al., 2014; Liu et al., 2015). The APOEe4 isoform appears to be more effective in blocking these receptors, leading to a loss of growth factor signaling and reduced cell growth, repair and metabolism, and additionally reduced inhibition of inflammation (Krasemann et al., 2017). It is easy to see how such actions could enhance the risk of developing AD, in particular if the growth factor insulin has lost its effectiveness. APOEe4 was even found to inhibit IR activity and enhance the uptake of the receptor in the cell (Zhao et al., 2017). When analyzing plasma samples of AD patients, it was shown that in APOEe4 allele carriers, microglia activation markers were different from those that carry the APOEe2 allele, and in non-demented controls. The marker CD68 was found to be increased in APOEe4 allele carriers, and this correlated with a poorer outcome in the Mini-Mental State Examination compared to carriers of the APOEe2 allele. APOEe2 allele carriers showed enhanced levels of Iba-1 microglia markers which correlated with a better performance in the in the Mini-Mental State Examination (Minett et al., 2016). Furthermore, APOEe4 carriers showed enhanced plasma levels of TNF-α and reduced Akt/PKB activity (Morris et al., 2018). These observations support the concept that AD progression is linked to reduced growth factor activity and a reduced inhibition of the inflammation response.

Another important risk factor for AD is the gene for TREM2, an immune phagocytic receptor expressed on brain microglia that regulates phagocytosis and the inflammation response. Homozygous mutations in TREM2 cause Nasu-Hakola disease, a rare recessive form of dementia (Jin et al., 2014). TREM2 is a main regulator of the activation of microglia in the inflammation response (Konishi and Kiyama, 2018). In carriers of TREM2 mutations, the inflammation response is much enhanced, and pro-inflammatory cytokines are increased (Roussos et al., 2015). Importantly, APOE and TREM2 interact in the regulation of the inflammation response, further emphasizing the key role of inflammation in AD progression (Morris et al., 2018; Shi and Holtzman, 2018).

Insulin reduces the chronic inflammation response by inhibiting secondary cell signaling induced by pro-inflammatory cytokines (Bamji-Mirza et al., 2014; Iloun et al., 2018; **Figure 1**).

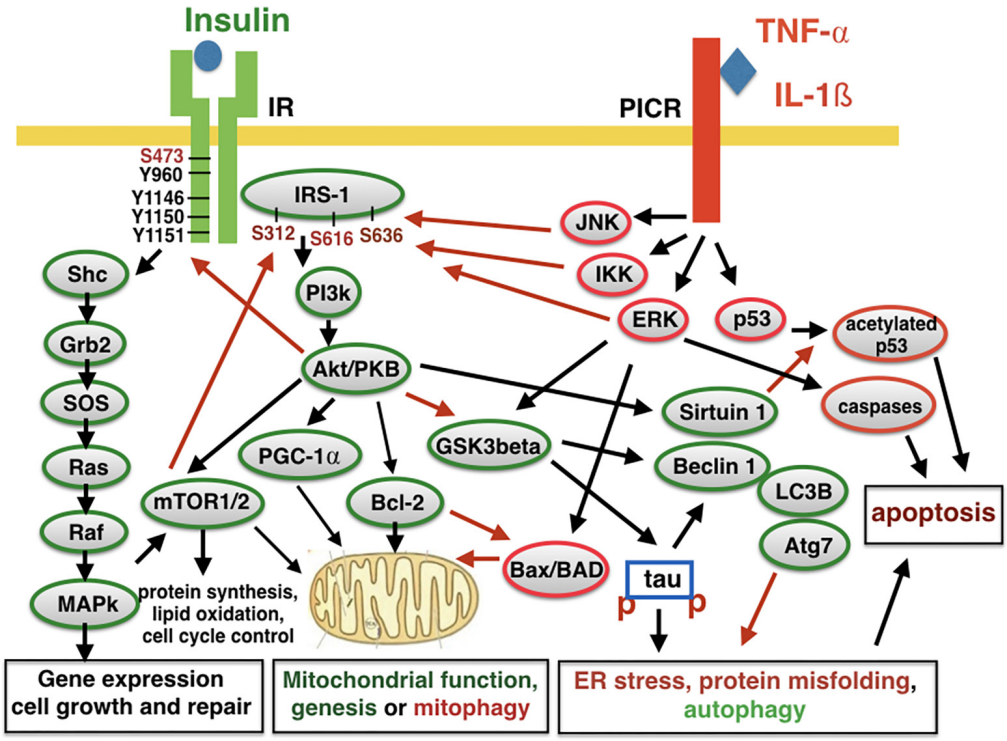

FIGURE 1 | Insulin signaling and pro-inflammatory signaling counteract each other. The activation of the insulin receptor (IR) leads to auto-phosphorylation and activation. Several other kinases can activate or inactivate the receptor. The insulin-receptor substrate 1 (IRS-1) also contains several phosphorylation sites that can activate or inhibit second messenger signaling. Downstream signaling activates key physiological processes such as energy utilization, mitochondrial function and replacement, protein synthesis, autophagy, and inhibiting autophagy. Activating pro-inflammatory cytokine receptors (PICR) counteracts these processes and enhances mitophagy, autophagy, and apoptosis. Kinases such as JNK and IKK can phosphorylate IRS-1 to inhibit insulin signaling and induce insulin de-sensitization. IL-1ß, interleukin 1ß; TNF-α, tumor necrosis factor a; JNK, NH2-terminal c-Jun kinase; IKK, inhibitor of kappa B kinase; ERK, extracellular regulated kinase; MAPK, mitogen activated protein kinase; SHP-2, Src-like homology 2(SH2) domain containing protein tyrosine phosphatase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; Grb2, growth factor receptor binding protein 2; SOS, son of sevenless protein; PI3K, phosphatidylinositol 3-kinase; IRS, insulin receptor substrate; PKB, protein kinase B,also known as Akt; Raf, regulation of alpha-fetoprotein; Ras, rat sarcoma virus peptide; LC3B, microtubule-associated proteins 1A/1B light chain 3B; Atg7, autophagy-related protein 7; Bcl-2, B-cell lymphoma 2; Bad, Bcl-2-associated death promoter; Bax, Bcl-2 associated X protein; Shc, Src homology collagen peptide. Red arrows, inhibiting activity; Black arrows, activating activity.

A de-sensitization of insulin signaling enhances the inflammation response and the de-sensitization observed in T2DM (Biessels et al., 2002), therefore, not only compromises growth factor signaling, and energy utilization in the brain, but also facilitates the chronic inflammation response.

Triggers for Chronic Inflammation in the Brain

The chronic inflammation response observed in neurodegenerative disorders is triggered initially and maintained by continuous stress to the system. Ordinarily, the inflammation response would be terminated after the initial event that triggered it had stopped. Due to the nature of neurodegenerative disorders, such as AD, the stressor persists and the inflammation response becomes chronic. Mutations in the APP gene are known to cause early-onset familial forms of AD (Hardy, 1997; Hutton et al., 1998; Blennow et al., 2006). The aggregated or misfolded amyloid protein that results from this can induce an inflammation response (Lourenco et al., 2013), most likely by activating Toll-like receptors (Clark and Vissel, 2018). In animal models that express human mutated APP, a chronic inflammation response is observed in the brain (McClean and Holscher, 2014a; Olmos-Alonso et al., 2016). The chronic exposure to such low-level inflammation stress could lead to AD. It is important to note in this context that the mutated APP gene does not cause AD but accelerates its development. This could explain why the removal of amyloid plaques from the brains of AD patients did not stop disease progression (Holmes et al., 2008). By the time the amyloid is removed, the chronic inflammation response has already been firmly established and continues to expose neurons to oxidative stress and pro-inflammatory cytokines. Auto-immune conditions are also under discussion as drivers for AD. It is straight-forward to see that an auto-immune response in the brain could start chronic inflammation responses which eventually lead to AD (Korczyn and Vakhapova, 2007; Lehrer and Rheinstein, 2015). The NIH decided to fund further investigations of this link with AD (McCarthy, 2014).

Viral infection is another possible way of inducing a chronic inflammation response in the brain. Several studies show that

viral infection can cause neurodegeneration and encephalitis if it infiltrates the brain (Damasio et al., 1989; Hogestyn et al., 2018). A recent study demonstrated that the Herpes virus was present in brain tissue of AD patients (Readhead et al., 2018). It is, however, not clear whether the virus was the cause of AD or if the infection occurred when the disease was already established, and the immune system had been compromised. It is, however, feasible that in some cases, the infection of the brain in vulnerable people induces a chronic inflammation response that can eventually lead to AD (Itzhaki et al., 2004a). The same model applies to infection with bacteria such as *Chlamydia pneumoniae*, which has been identified in brain tissue of people with AD (Itzhaki et al., 2004b).

High blood pressure is another risk factor for developing AD (Marfany et al., 2018). Again, enhanced inflammation in the brain has been found in people with high blood pressure (Wenzel, 2018). Stroke and hemorrhage in the brain is another risk factor for AD. The induction of an inflammation response in the brain has been well established (de Oliveira Manoel and Macdonald, 2018).

T2DM not only desensitizes insulin signaling in the brain (Blazquez et al., 2014), it also enhances inflammation markers such as levels of pro-inflammatory cytokines (Herder et al., 2013; Illien-Junger et al., 2013; Denver et al., 2018). Importantly, activated microglia are also found in the brains of people with T2DM, paving the way for chronic inflammation in the CNS that could lead to AD (Maldonado-Ruiz et al., 2017).

This short list of potential risk factors for AD that trigger an inflammation response is by no means complete. It demonstrates that there may be many initial triggers that could result in the development of AD in later life, depending on additional factors such as the individual vulnerability to these triggers and the inability of the brain to bring the inflammation response under control. For example, possession of the APOEe4 allele increases the vulnerability to viral infections in the brain (Itzhaki et al., 2004b).

RE-SENSITIZING INSULIN SIGNALING IN THE BRAIN TO PREVENT AD

Treating AD Patients With Insulin

As insulin signaling has been identified as a feature of AD pathology, several strategies have been tested to find out if re-sensitizing insulin signaling in the brains of AD patients may be promising. Treating patients with insulin via nasal application to increase transfer into the brain, while reducing peripheral effects of insulin, has shown very promising effects in several clinical trials (Craft et al., 2013; Freiherr et al., 2013).

A pilot study testing nasal application of insulin in MCI/AD patients tested 26 memory-impaired subjects and 35 controls. The study had a double blind and randomized patient allocation design and a placebo group. Importantly, the insulin treatment had no effect on plasma insulin or glucose levels. Insulin treatment showed a better effect in memory-impaired non-APOEe4 carriers than for memory-impaired APOEe4 carriers and control subjects (Reger et al., 2006). A second double blind pilot study tested 24 AD/MCI patients. The insulin-treated group showed improved memory for verbal information after a delay compared to the placebo group, and furthermore improved the amyloid 40/42 ratio (Reger et al., 2008b).

In a third study, 33 AD/MCI patients and 59 control subjects received five intranasal treatments of insulin or placebo which improved recall of verbal memory in memory-impaired non-APOEe4 carriers. However, memory-impaired APOEe4 carriers displayed a decline in verbal memory. Drug treatment also improved plasma amyloid-beta levels in memory-impaired subjects and controls depending on APOE genotype status (Reger et al., 2008a). These results demonstrate a complex interaction between insulin treatment and APOEe allele status. As APOE plays an important role in inflammation, and chronic inflammation is an important aspect of AD disease progression, it is easy to see that different alleles can have facilitating or inhibiting effects on AD development. APOEe4 appears to enhance the inflammation response (McGeer et al., 1997). It acts in conjunction with TREM2 to regulate microglia activation in the brain (Morris et al., 2018; Shi and Holtzman, 2018). This could explain why carriers of the APOEe4 allele have an increased risk of developing AD. As insulin can also activate an inflammation response at higher concentrations, most likely as a negative feedback mechanism to avoid excessive energy utilization (Tsatsoulis et al., 2013; Maldonado-Ruiz et al., 2017), the dosing of insulin and the state of insulin de-sensitization in the AD patients will be crucial to determine the outcome of insulin treatment.

A larger randomized, double-blind, placebo-controlled clinical pilot trial tested 104 patients with either MCI or mild to moderate AD. Patients received either placebo or one of two doses of insulin for 4 months. Primary measures consisted of delayed story recall score and the Dementia Severity Rating Scale score, and secondary measures included the Alzheimer Disease's Assessment Scale-cognitive subscale (ADAS-cog) score and the Alzheimer's Disease Cooperative Study-activities of daily living (ADCS-ADL) scale. ^{18}FDG-PET brain scans were conducted before and after treatment and showed a clear protection of cortical activity in AD patients. This scan measures glucose uptake and activity of neurons in the brain. Both insulin doses preserved general cognition and functional abilities assessed by care givers. Episodic memory was improved and changes were still present 2 months after cessation of treatment (Craft et al., 2012).

Another trial in AD/MCI patients tested the insulin analog detemir (Levemir) for 3 weeks. Treated patients improved in memory performance compared to a placebo group, but only if they had high levels of insulin resistance at baseline. Detemir is more effective than insulin and remains in the body for up to 24 h. Surprisingly, neither dose of insulin was more effective than placebo in memory tests. However, when patients in each treatment arm were grouped into those with low versus high insulin resistance, both groups differed from placebo controls. Patients with high insulin resistance showed an improvement in the memory tests when treated with detemir, compared to an increase of less than 0.1 point in the placebo patients with high insulin resistance. The patients with low insulin resistance

treated with detemir had a bigger decrease in memory tests than the placebo group. Carriers of the APOEe4 allele were benefiting from detemir treatment, while non-carriers showed worsened memory compared to placebo (Claxton et al., 2013, 2015; Craft et al., 2013). A double blind, placebo-controlled study tested the effects of 4 months of nasal insulin or detemir treatment. Interestingly, the detemir group did not show improvements, while the insulin-treated group showed preserved memory performances and brain tissue volumes in MRI scans. An improved ration of phosphorylated tau to amyloid 42 was also observed in CSF samples (Craft et al., 2017).

These different clinical trials demonstrate that overcoming insulin resistance in the brain has protective effects in MCI/AD patients and shows improvements in cognition and relevant biomarkers. It is a proof of concept that insulin desensitization is instrumental in driving cognitive impairments in AD, and that an improvement of insulin signaling has genuine benefits.

Strategies to Re-sensitize Insulin Signaling in the Brain

Metformin

Several treatment strategies exist to improve insulin signaling in people with T2DM. A standard drug prescribed to patients is metformin, which can re-sensitize insulin signaling in diabetes. However, when comparing metformin with GLP-1 receptor agonists in animal models of neurodegeneration, metformin is far less protective (Gault and Holscher, 2018). In a clinical trial testing metformin in patients with AD, the drug showed no protective effect. Treatment for 12 months only showed a very small improvement in a recall test with no changes in ADAS-cog or in ^{18}FDG-PET brain scans (Luchsinger et al., 2016). While metformin is widely used in the clinic, it is actually not a very potent drug and more effective drugs will have to be given as T2DM progresses (Lentferink et al., 2018). Epidemiological studies report conflicting results with some showing a reduction of the risk to develop AD when taken over years (Hsu et al., 2011; Ng et al., 2014), while other studies showed an increase in the risk of developing AD after long-term use (Imfeld et al., 2012; Kuan et al., 2017). In a direct comparison with other drugs that enhance insulin sensitivity, metformin was inferior to more potent drugs such as GLP-1 analogs (Lennox et al., 2014). The reason for this may be that metformin only activates the AMP kinase, which is activated during starvation, but not the growth-factor associated PI3kinase (Turban et al., 2012). AMP kinase activates key survival genes while blocking energy expenditure on routine housekeeping activity in the cell (Hardie et al., 2012). PI3kinase, in contrast, is activated by growth factors such as insulin and IGF-1 and activates all housekeeping genes, ensuring cell growth, and repair (Talbot et al., 2012; Craft et al., 2013; Holscher, 2014). Therefore, it is easy to see that metformin is only helpful for the short term.

Leptin

Leptin is a hormone and growth factor that regulates energy utilization in cells, similarly to insulin. Both leptin and the leptin receptor are expressed in the brain where they play a range of roles. Importantly, some leptin receptor isoforms are linked to second messenger signaling cascades that also activate IRS, PI3k, and Akt/PKB, as insulin receptors do (see **Figure 1**), and therefore, can control energy utilization and cell growth on a similar level to insulin (Sweeney, 2002; Marwarha and Ghribi, 2012; Paz-Filho et al., 2012). Leptin can re-sensitize insulin signaling by interacting with the IR and signaling pathway (Fruhbeck, 2006; German et al., 2010; Mantzoros et al., 2011; Paz-Filho et al., 2012). Leptin can enhance memory formation and upregulate synaptic plasticity in the hippocampus (Harvey et al., 2006). In animal models of AD, leptin showed protective effects, and improved performances in learning tasks (Greco et al., 2010; Perez-Gonzalez et al., 2011; Sharma and Hölscher, 2014).

As leptin is linked to glucose uptake and energy utilization in cells, it is not surprising that leptin signaling desensitizes in T2DM, just as insulin signaling does (Kautzky-Willer et al., 2001; Carey et al., 2003; Cummings, 2013; Sharma and Hölscher, 2014). This observation convinced researchers studying T2DM not to develop drugs that act on the leptin receptor as potential treatments for diabetes (Mantzoros et al., 2011; Chou and Perry, 2013). There is also evidence that leptin signaling is de-sensitized in AD patients, most likely driven by the chronic inflammatory response in the brain (Lieb et al., 2009; Clark et al., 2011; Bonda et al., 2014; Khemka et al., 2014). This would make it difficult to use leptin to re-sensitize insulin signaling in the brains of AD. One proposal to circumvent the problem is to co-apply different hormone analogs that can re-sensitize leptin signaling (Sadry and Drucker, 2013).

The story does not end here, though. Leptin also plays a role in pro-inflammatory signaling. The receptor is expressed on cell types that play key roles in the inflammation response (Procaccini et al., 2012). As a hormone and growth factor, it stimulates the activity of macrophages, neutrophils, and natural killer cells (Matarese et al., 2005). It also activates the proliferation of T helper cells and the differentiation of CD4+ T-cells into inflammatory Th-17 cells (Lord et al., 1998). The transgenic models of obesity that have mutations in leptin and the leptin receptor genes, ob/ob and db/db, show enhanced inflammatory responses (Lord et al., 2001). In the animal model of multiple sclerosis (MS), an auto-immune response is induced. When trying to induce the auto-immune response in ob/ob mice, the response is much reduced, and low levels of pro-inflammatory cytokines are expressed (Matarese et al., 2001b). When replacing the missing leptin in the ob/ob mice, a full-blown auto-immune reaction is induced, and levels of pro-inflammatory cytokines are much increased (Matarese et al., 2001a). Removing leptin in wild type mice also makes them less susceptible to developing the MS auto-immune disease phenotype (Ouyang et al., 2014). As chronic inflammation plays a key role in AD, it is therefore of benefit for leptin signaling to be de-sensitized. Therefore, leptin treatment may not be beneficial as a treatment for neurodegenerative disorders (de Candia and Matarese, 2018).

Glucagon-Like Peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP)

Glucagon-like peptide 1 and Glucose-dependent insulinotropic polypeptide are incretin hormones that signal high energy status in the body, similar to insulin and leptin (Lund et al., 2011).

They activate membrane standing G-protein coupled receptors that are members of the glucagon-type growth factor receptor family. Classic growth factor signaling cascades are activated by the receptors, including the cAMP-PKA-CREB pathway that enhances cell growth, cell repair and expression of insulin, and insulin receptors (Baggio and Drucker, 2007; Doyle and Egan, 2007; Hölscher, 2016). The PI3k-Akt/PKB-mTOR pathway is also activated, compensating for the loss of insulin signaling (Hölscher, 2018). AMPk is also activated by the receptors (Chang et al., 2018). GLP-1 enhances insulin release making it an attractive treatment for T2DM (Baggio and Drucker, 2007; Doyle and Egan, 2007; Long-Smith et al., 2013). GLP-1 receptor agonists have been developed that have an enhanced biological half-life in the blood stream. Several are currently on the market to treat diabetes (Campbell and Drucker, 2013; Elkinson and Keating, 2013; Lean et al., 2014). GLP-1 mimetics re-sensitize insulin signaling in the brains of animal models of AD (Bomfim et al., 2012; Long-Smith et al., 2013; Shi et al., 2017) and in patients with T2DM (Zander et al., 2002).

Glucagon-like peptide 1 mimetics showed clear neuroprotective effects in several animal models of AD (Li et al., 2010). The GLP-1 mimetic Liraglutide reduced AD hallmarks such as amyloid plaque load, memory loss, synapse loss, impaired synaptic transmission (LTP), and the chronic inflammation response in the brain (McClean et al., 2011, 2015; McClean and Holscher, 2014a). The GLP-1 mimetic lixisenatide showed similar neuroprotective effects in the APP/PS1 mouse model of AD (McClean and Holscher, 2014b). Furthermore, liraglutide reduced tangles and phosphorylated tau levels in the human P301L mutated tau gene expressing mouse, a model of fronto-temporal lobe dementia (Hansen et al., 2015). Liraglutide reduced insulin de-sensitization and reduced the inflammation response caused by the injection of amyloid oligomers into the brains of cynomologous monkeys (Lourenco et al., 2013; Batista et al., 2018). GLP-1 mimetics protect mitochondrial activity and enhance mitochondrial genesis by reducing apoptotic BAX/BAD signaling and by increasing pro-mitochondrial Bcl-2 and PGC-1a signaling (Li et al., 2016a; Shi et al., 2017; Chang et al., 2018; Wang et al., 2018). Importantly, GLP-1 mimetics protect against ER stress and normalize autophagy impairments found in neurodegenerative disorders, which can explain how amyloid or tau aggregates are removed by the activation of the GLP-1 receptor (Sharma et al., 2013; Panagaki et al., 2017).

A pilot clinical trial in AD patients demonstrated an increased glucose uptake in the brain after treatment with liraglutide (Gejl et al., 2016). Unfortunately, the trial was underpowered and did not show any results in other measures. A phase II clinical trial with 200 AD patients is currently ongoing. Patient recruitment has finished, and the results should become available late 2019 (clinical trial identifier NCT01843075). In a double-blind, placebo-controlled phase II clinical trial in PD patients, the GLP-1 receptor agonist exendin-4 showed good protective effects compared to placebo, even after 3 months of wash-out of the drug (Athauda et al., 2017). Further clinical trials testing other GLP-1 mimetics are currently ongoing.

The sister hormone GIP also has neuroprotective properties in different animal models of neurodegenerative disorders. Stable analogs such as D-ala^2-GIP or N-glyc-GIP reversed impairments of synaptic plasticity induced by amyloid (Gault and Holscher, 2008). The GIP analog D-Ala^2-GIP showed neuroprotective effects in the APP/PS1 mouse model of AD by protecting memory formation, synaptic plasticity, reducing the amyloid plaque load and the chronic inflammation response in the brain, reducing oxidative stress, and normalizing neurogenesis in the dentate gyrus (Duffy and Holscher, 2013; Faivre and Holscher, 2013a,b). GIP injection ip., had protective effects on spatial learning in memory tasks and also reduced plaque formation and amyloid load in a different AD mouse model (Figueiredo et al., 2010). See (Ji et al., 2016; Verma et al., 2018) for a review.

In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, GIP analogs showed good protective effects. Motor activity was partly rescued, and the number of dopaminergic neurons in the substantia nigra was increased. The cAMP/PKA/CREB growth factor second messenger pathway was shown to be activated by the drug. The increased levels of expression of alpha-synuclein in the brain induced by MPTP were reduced by the drug. In addition, drug treatment reduced chronic neuroinflammation, oxidative stress and lipid peroxidation, and increased the expression of the growth factor BDNF (Ji et al., 2016; Li et al., 2016c; Li Y. et al., 2017; Verma et al., 2017, 2018).

Novel dual GLP-1/GIP receptor agonists also show good effects in different animal models of neurodegenerative disorder (Hölscher, 2018).

Ghrelin

Ghrelin is a member of the hormones and growth factors that signal energy status in the body. It is released by stomach cells during times of low food availability. It is considered the main factor that drives the health-improving effects of fasting/caloric restriction (Gomez et al., 2009; Bayliss et al., 2016b). Ghrelin does not re-sensitize insulin as it is active at low plasma glucose levels, while insulin works "against" ghrelin and is only active at high glucose levels. However, ghrelin activates a range of growth-factor signaling pathways that can compensate for a loss of insulin signaling. Ghrelin has been shown to have impressive protective effects in animal models of Parkinson's disease. Administration of ghrelin reduced the neurodegeneration observed in the MPTP model of PD. Ghrelin reduced the MPTP-induced loss of substantia nigra (SN) dopamine neurons and striatal dopamine turnover. Ghrelin activated AMPk and ACC- driven lipid-oxidation in the striatum. Mitochondrial activity and genesis were improved. Importantly, the chronic inflammation response in the brain was much reduced by the drug (Bayliss et al., 2016a,b; Morgan et al., 2018). These impressive effects make ghrelin a candidate to be a novel treatment for PD. However, there are downsides of this strategy.

One of the main modes of action is the activation of AMP-kinase (AMPk) by the Ghrelin receptor. AMPk is a kinase that is activated under conditions of low energy, such as low cellular ATP and high AMP levels. AMPk is a key regulator of a range of physiological processes that enhance cell survival. Glucose uptake and oxidation, as well as activation of fat reserves, uptake of fatty acids and lipid oxidation to generate ATP, are increased. Importantly, AMPK upregulates genes involved

in oxidative metabolism and oxidative stress resistance by regulating transcription factors of the abnormal dauer formation 16 (DAF-16)/forkhead box O (FOXO) family. This enables cells to deal with enhanced oxidative stress, which will increase in conditions of low energy and failing mitochondrial activity (Halliwell, 2006; Hardie et al., 2012). In brief, catabolic pathways are much enhanced by ghrelin signaling to ensure sufficient energy supply for cells, while anabolic pathways that are energy demanding are reduced. These include protein synthesis and ribosomal RNA (rRNA) synthesis, triglyceride synthesis, cholesterol synthesis, transcription of gluconeogenic enzymes, and others (Hardie et al., 2012). It is easy to see that such a strategy will improve survival in lean times by focusing energy expenditure on the core processes for survival. However, it is not sensible to inhibit protein synthesis over long periods of time. In particular, in neurodegenerative disorders, repair mechanisms that require de novo protein synthesis are of major importance. A first clinical trial of a ghrelin analog also points to major issues that cannot be easily inferred from animal studies. The survival time of ghrelin in the blood stream is short, but long-acting protease-resistant analogs have been developed for the clinic. Relamorelin is a pentapeptide and analog of ghrelin with improved potency and pharmacokinetics. In humans, relamorelin produces increases in plasma growth hormone, prolactin, and cortisol levels, and increases appetite. A multi- centre, randomized, double-blind, placebo-controlled study of relamorelin in patients with PD had to be terminated, as most patients experiencedchronic and severe constipation, which is another side effect of ghrelin. Only 18 out of 56 subjects completed the trial, in part because of multiple partially complete bowel movements in constipated PD patients, which made many subjects ineligible for further participation (Parkinson Study Group, 2017). Such drastic side effects do not bode well, and long-term effects of drug treatment have yet to be investigated. However, the investigation of the effects of ghrelin enriched out knowledge of underlying mechanisms of neuronal pathology and neuronal protection, which may be utilized in other forms and treatment strategies.

DPP-IV Inhibitors

Currently, inhibitors of the enzyme protease dipeptidyl-peptidase IV (DPP-IV) are on the market treat T2DM (Ahren, 2007). DPP-IV degrades GLP-1, GIP and other peptide hormones. Inhibition of DPP-IV therefore can enhance the blood levels of these hormones and can treat T2DM effectively. Inhibitors of DPP-IV also have protective effects in animal models of AD (Li et al., 2016b). However, DPP-IV inhibitors are small molecules that do not readily cross the BBB. The DPP-IV inhibitor saxagliptin (SAX) also showed good neuroprotective effects in the ICV. STZ animal model of AD that models insulin desensitization in the brain. Here, animals were orally administered at 0.25, 0.5, or 1 mg/kg for 60 days after treatment with STZ. The highest dose of 1mg/kg showed good protection in memory tests and lowered key biomarkers for AD and cellular stress (Kosaraju et al., 2013). In a similar study from the same lab, Vildagliptin also demonstrated neuroprotective effects. However, the effective dose was more than 10 times higher with lower protective efficiency, suggesting that Vildagliptin does not cross the BBB as readily as SAX. In addition, the protective effects of DPP-IV inhibitors in animal models are limited compared to GLP-1 receptor agonists (Abdelsalam and Safar, 2015; Darsalia et al., 2016). This is most likely due to the fact that DPP-IV inhibitors work indirectly by reducing peptide hormone cleavage, while GLP-1 receptor agonists act directly on the receptors.

CONCLUSION

The evidence presented here demonstrates that insulin de-sensitization contributes to the disease progression in AD, and that the use of insulin re-sensitizing agents show promise to make a difference and to reduce disease progression. First clinical trials have shown promising results and suggest that the protective effects observed in animal studies translate to the clinic. This strategy shows great promise and hopefully will produce effective novel treatments for AD and other chronic progressive neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

CH wrote the manuscript.

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Conflict of Interest Statement: CH is a named inventor on several patent applications that cover the use of dual GLP-1 and GIP receptor agonists to treat neurodegenerative disorders.

frontiers in Aging Neuroscience

HYPOTHESIS AND THEORY
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Involvement of *microRNA-34a* in Age-Related Susceptibility to Oxidative Stress in ARPE-19 Cells by Targeting the Silent Mating Type Information Regulation 2 Homolog 1/p66shc Pathway: Implications for Age-Related Macular Degeneration

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The aging retinal pigment epithelium and oxidative stress, mediated by reactive oxygen species (ROS) accumulation, have been implicated in the mechanisms of age-related macular degeneration (AMD). The expression level of the adapter protein p66shc, a key protein that regulates cellular oxidative stress, is relatively low under normal conditions because of the effects of silent mating type information regulation 2 homolog 1 (SIRT1) on the binding of fully deacetylated histone H3' to the *p66shc* promoter region, thus inhibiting p66shc transcription and expression. The equilibrium between SIRT1 and p66shc is disrupted in the presence of various stresses, including AMD. As a major target gene, *SIRT1* is regulated by *microRNA-34a* (*miR-34a*), and overexpression of *miR-34a* results in significant inhibition of post-transcriptional expression of *SIRT1*. Furthermore, our recent studies demonstrated that *miR-34a* is significantly upregulated, accompanied by reduced tolerance to oxidative stress in hydrogen peroxide-induced prematurely senescent ARPE-19 cells. Moreover, the expression of SIRT1 is decreased, whereas that of p66shc is increased in these cells. Accordingly, *miR-34a* may play a key role in age-related susceptibility to oxidative stress in ARPE-19 cells by targeting the SIRT1/p66shc pathway, leading to AMD. In this review article, we discuss the functions of *miR-34a* in modulating the SIRT1/p66shc pathway in age-related conditions, including AMD.

Keywords: microRNA-34a, silent mating type information regulation 2 homolog 1, p66shc, age-related macular degeneration, oxidative stress, premature senescence

INTRODUCTION

Age-related macular degeneration (AMD), a leading cause of vision impairment in the elderly, is a multifactorial disease that involves age, gene variants of complement regulatory proteins, and smoking. With the increased aging of society, the number of patients with AMD is increasing, making this condition an important public health problem worldwide (Lim et al., 2012). AMD accounts for 54% of blindness in Caucasian Americana (Congdon et al., 2004), and a systematic review of data from an epidemiological survey of AMD prevalence found that the prevalence of any AMD ranged from 2.44% in people ages 45–49 years to 18.98% in people ages 85–89 years (Song et al., 2017). In a global meta-analysis, the overall prevalence of any AMD was 8.69% (Wong et al., 2014), and approximately 1.7 million people in the United States were affected by AMD, causing severe visual impairment in adults older than 65 years (Abbasi, 2018). Approximately 25.0% of eyes considered normal based on dilated eye examinations by primary eye care physicians (Neely et al., 2017); thus, the incidence of AMD is higher. The characteristic lesions of AMD are drusen, which are visible clinically in both the macula and retinal periphery (Mitchell et al., 2018). As AMD progresses, it can develop into two distinct forms of late AMD: "dry," atrophic AMD, characterized by retinal pigment epithelium (RPE) senescence and geographic RPE loss, and "wet," neovascular AMD, characterized by abnormal growth of choroidal new vessels (Zhu et al., 2009). Intraretinal or subretinal leakage, hemorrhage, and RPE detachments may occur with neovascular AMD, resulting in a rapid decline in vision. However, RPE atrophy or geographic atrophy may occur with late phase of dry AMD, resulting in a gradual vision decrease (Hallak et al., 2019).

Although the development of anti-vascular endothelial growth factor (VEGF) drugs has revolutionized the treatment of wet AMD (Martin et al., 2011), some patients show poor responses to these drugs. Additionally, there are few effective treatments for dry AMD. Therefore, current studies have focused on exploring the pathogenesis of AMD and identifying targets for early intervention and treatment of AMD.

Although the exact pathogenesis of AMD is not fully understood, a number of metabolic abnormalities have been shown to be associated with the development of this disease (Ding et al., 2009). For example, oxidative stress and oxidative stress-induced inflammation were found to initiate AMD in one study (Hollyfield et al., 2008).

In this review article, we provide a discussion of recent literature describing the mechanisms of AMD development, with a focus on RPE senescence, silent mating type information regulation 2 homolog 1 (SIRT1) signaling, and microRNA (miRNA) activity.

RELATIONSHIP BETWEEN RPE SENESCENCE AND AMD

Age is the most important risk factor for AMD (Curcio et al., 2009). More than 10% of older people over 80 suffer from advanced AMD (Smith et al., 2001). Indeed, with the increasing age, the organs and tissues of the whole body, including retinal tissue, showed decreased function. The RPE is a highly specialized epithelial cell layer with polarity that interacts with photoreceptors on its apical side and with Bruch's membrane and the choriocapillaris on its basal side (Datta et al., 2017). The RPE functions in phagocytosis of the photoreceptor outer segment and thereby plays an important role in maintaining the normal physiological function of photoreceptors.

With aging and the gradual accumulation of environmental stresses, RPE cells gradually show dysfunction, including decreased phagocytosis, lipofuscin deposition, and drusen formation. These morphological and functional changes in the outer retina induced by RPE degeneration play an important role in the pathogenesis of AMD.

Notably, oxidative stress induces premature senescence in RPE cells, leading to an imbalance in VEGF and complement factor h, and may be a main player in the induction and progression of AMD (Marazita et al., 2016). As shown in

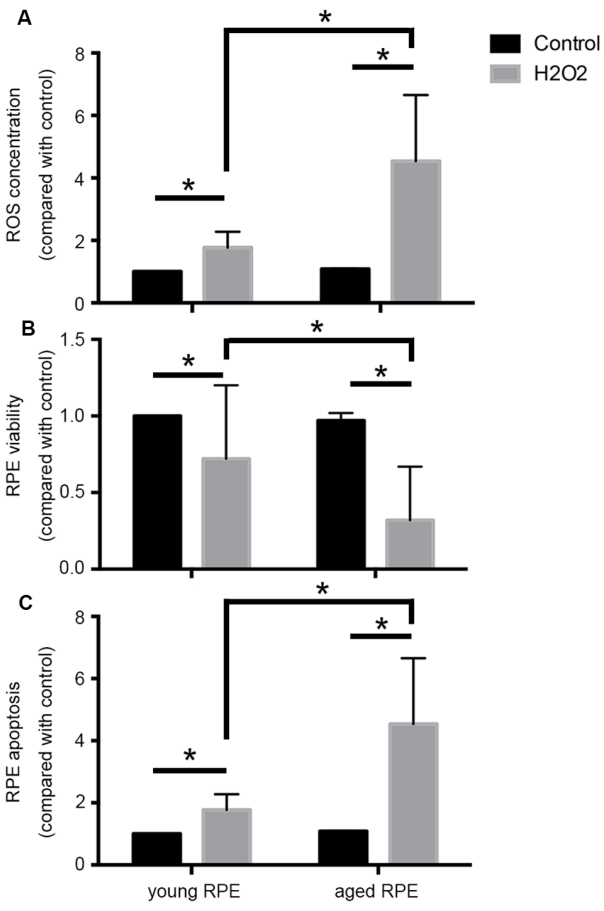

FIGURE 1 | Aging induced susceptibility to oxidative stress in ARPE-19 cells. Aged ARPE-19 cells produced more reactive oxygen species (ROS; **A**), and cell viability decreased **(B)**, whereas apoptosis increased **(C)** significantly after hydrogen peroxide stimulation when compared with the effects in young ARPE-19 cells. *$p < 0.05$ significantly different when compared within two groups.

Figure 1, in our preliminary experiments, we found that aged adult human retinal pigment epithelial (ARPE-19) cells produced more reactive oxygen species (ROS) and that cell viability decreased and apoptosis increased significantly after hydrogen peroxide stimulation when compared with the results in young cells, providing support for the age-related susceptibility of ARPE-19 cells to oxidative stress.

RELATIONSHIPS AMONG *miR-34a*, RPE SENESCENCE, AND AMD PATHOGENESIS

miRNAs are small noncoding RNA molecules (∼20–24 nucleotides in length) found in all cell types. These molecules lead to RNA silencing and post-transcriptional regulation of target gene expression, thus affecting cellular function and determining cell fate. Many miRNAs are differentially expressed in the circulation in patients with AMD (Ren et al., 2017). Additionally, significant changes in miNRA expression have been observed in retinal tissues from patients with AMD by bioinformatics and microarray technology. These miRNAs may be involved in the pathological processes of AMD by targeting downstream transcription factors. However, the specific regulatory mechanisms are still unclear (Berber et al., 2017).

As a p53-regulated miRNA, *miR-34a* was first discovered in studies of cancer. The activation of *miR-34a* promotes cell apoptosis and inhibits tumorigenesis. Recently, *miR-34a* has been shown to be closely related to cellular senescence in tissues. Notably, *miR-34a* is significantly upregulated in aged hearts and can promote the senescence of cardiac myocytes and decrease systolic function by inhibiting the expression of protein phosphatase 1 nuclear-targeting subunit and inducing telomere shortening (Boon et al., 2013). In contrast, *miR-34a* can also promote cellular senescence to maintain normal physiological function and avoid unlimited cell proliferation. Indeed, *miR-34a* regulates telomerase activity in hepatocellular carcinoma cells and promotes cellular senescence by targeting the FoxM1/c-Myc pathway (Xu et al., 2015). *miR-34a* is also a pivotal regulator of aging. For example, some drugs can regulate aging by affecting the expression of *miR-34a* and activating related signal pathways (Ye et al., 2016; Guo et al., 2017; He et al., 2017). Additionally, there was an age-dependent increase in *miR-34a* expression in the posterior pole of the mouse eye, and DNA damage in the mitochondria in retinal cells and RPE cells was found to be related to age and upregulation of *miR-34a* expression (Smit-McBride et al., 2014). Thus, *miR-34a* may play an important role in the pathophysiological mechanisms of cell senescence and apoptosis in the retina and in RPE cells in the aging eye.

In our previous studies, we evaluated the differential expression of *miR-34a* in the vitreous humor, collected during pars plana vitrectomy, from young patients without AMD, elderly patients without AMD, and patients with AMD. As shown in **Figure 2A**, the expression of *miR-34a* in the vitreous humor of the three groups of patients was increased, and there was a positive correlation between the expression of *miR-34a* and age (**Figure 2B**), as demonstrated by Pearson correlation analysis.

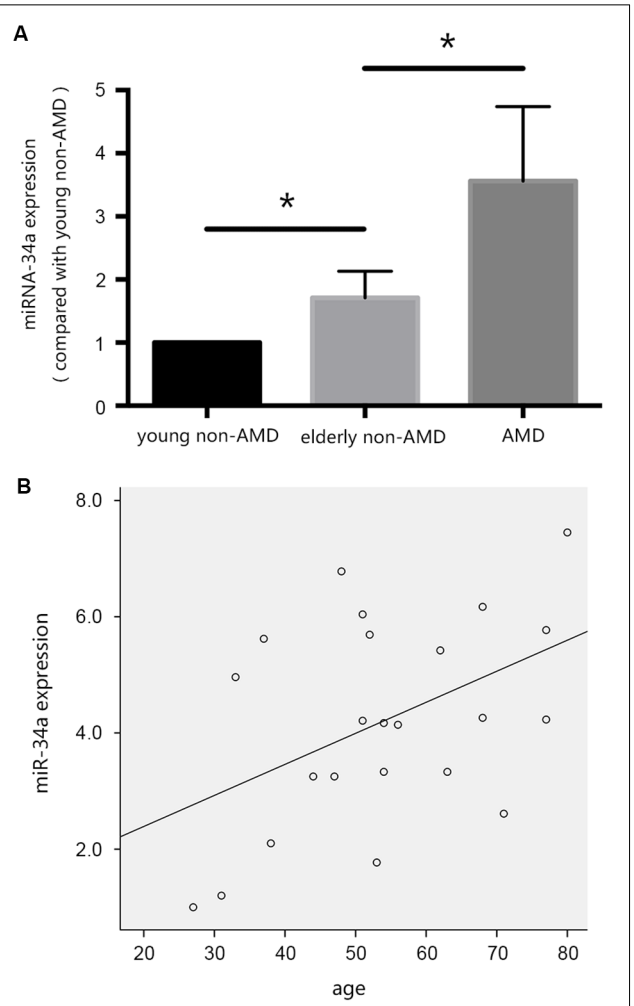

FIGURE 2 | *miR-34a* may be involved in the pathogenesis of age-related macular degeneration (AMD). **(A)** The expression of *miR-34a* in the vitreous humor of the three groups of patients was increased. $^*p < 0.05$ significantly different when compared within two groups. **(B)** Scatter plot showing the association between *miR-34a* expression and age. Note that there was a positive relationship between the expression of *miR-34a* and age ($r = 0.457$, $p = 0.029$).

These results indicated that *miR-34a* may be involved in RPE aging and the pathogenesis of AMD.

THE SIRT1/p66shc PATHWAY AND RESISTANCE TO OXIDATIVE STRESS

SIRT1, as an NAD-dependent histone deacetylase in mammals (Vaziri et al., 2001), is a homolog of sir2 in lower organisms and plays important roles in various biological activities, such as the cell cycle, apoptosis, DNA repair, and gene silencing. The fundamental mechanisms through which SIRT1 regulates the expression of target genes involve histone H3 deacetylation of the promoter or enhancer (Oppenheimer et al., 2014; Hsu et al., 2016).

p66shc is widely expressed in vertebrates and is a key protein involved in intracellular regulation of oxidative stress and the

life cycle. This protein induces cellular oxidative damage by producing a large number of ROS, mainly through the oxidation of cytochrome c in the mitochondria, and by inhibiting the elimination of ROS. Moreover, deletion of the *p66shc* gene can prolong the life span of mice by 30% *via* a mechanism involving enhancement of the resistance of mice to oxidative stress (Migliaccio et al., 1999).

Notably, SIRT1 can target the promoter region of *p66shc* (−508 to −250 bp), resulting in a decrease in the acetylation of histone H3 bound to this region. *p66shc* transcription is therefore inhibited, reducing tissue injury caused by oxidative stress. In acute ethanol-induced liver injury, the expression of p66shc was negatively correlated with changes in SIRT1 expression and decreased SIRT1 expression by RNA interference or nicotine significantly increased the expression of p66Shc (Tian et al., 2016). Therefore, the SIRT1/p66shc pathway may play important roles in blocking the effects of oxidative stress.

Many studies have shown that activation of SIRT1 may delay senescence in various tissues (Han et al., 2016; Guo et al., 2017; Hekmatimoghaddam et al., 2017), whereas inhibition of SIRT1 activity can cause premature senescence (Volonte et al., 2015), resulting in damage to cells caused by decreased tolerance of senescent cells to stress (Chuang et al., 2017). Additionally, the effects of SIRT1 on senescence are partly due to the regulation of *miR-34a* (Yang et al., 2013), although these effects have not been reported in retinal tissues and RPE cells. *miR-34a* was found to specifically bind with *SIRT1* mRNA in the 3′ untranslated region by bioinformatics prediction websites, such as pitcar, targetscan, Miranda, and microRNA.org.

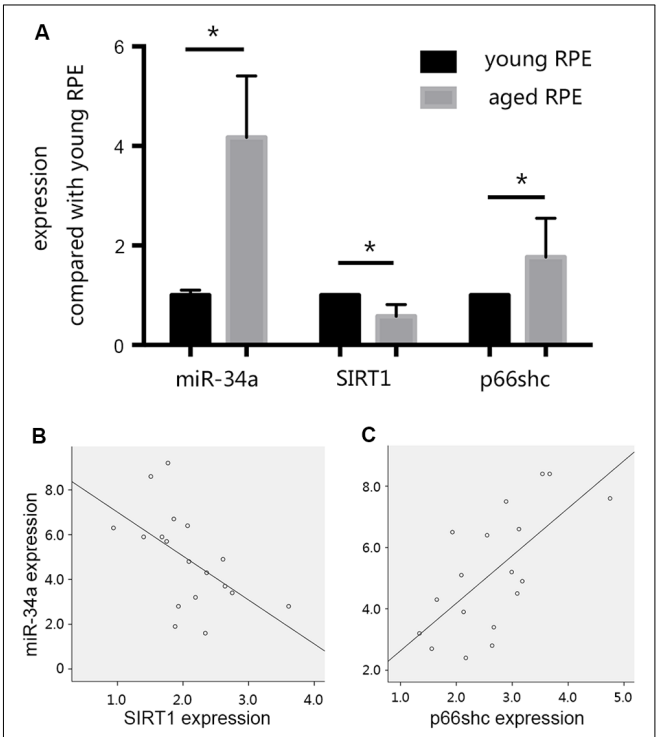

FIGURE 3 | Changes in the expression levels of *miR-34a*, silent mating type information regulation 2 homolog 1 (*SIRT1*), and *p66shc*. **(A)** The expression of *SIRT1* decreased, and the expression of *p66shc* increased in aged ARPE-19 cells, consistent with the increase in *miR-34a* expression. $^*p < 0.05$ significantly different when compared within two groups. **(B)** Scatter plot showing the association between *miR-34a* expression and *SIRT1* expression in ARPE-19 cells. Note that there was an inverse relationship between these targets ($r = -0.549$, $p = 0.018$). **(C)** Scatter plot showing the association between *miR-34a* expression and *p66shc* expression in ARPE-19 cells. Note that there was a positive relationship between these targets ($r = 0.666$, $p = 0.003$).

In our preliminary experiments, we established an RPE premature senescence model by hydrogen peroxide stimulation as described previously (Aryan et al., 2016). ARPE19 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). At passage 11, cells were seeded in 24-well plates at a density of 5×10^4 cells/well, and were routinely cultured in 95% air and 5% CO_2 at 37°C in DMEM/F12 medium containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. After 24 h of incubation, the growth medium was removed and replaced with serum-free medium (SFM). The medium of selected wells was changed to SFM + 0.3 mmol/L hydrogen peroxide 24 h later. After 90 min of hydrogen peroxide treatment, the medium in selected wells was changed to 1 ml of DMEM + 10% FBS for the subsequent study on cell senescence. The effect of hydrogen peroxide on retinal pigment epithelial cell senescence was investigated by S-Beta Galactosidase staining 24 h after treatment with hydrogen peroxide. We found that when compared with young RPE cells, the expression of SIRT1 decreased, whereas the expression of p66shc increased in aged RPE cells, consistent with the increase in *miR-34a* expression (**Figure 3A**). A reverse correlation between the expression of *SIRT1* and *miR-34a* (**Figure 3B**) and a positive correlation between the expression of *p66shc* and *miR-34a* (**Figure 3C**) were found by Pearson correlation analysis, suggesting that *miR-34a* may directly inhibit the expression of SIRT1 in aged RPE cells, resulting in a decrease in the inhibitory effect on downstream p66shc and an increase in the expression of p66shc.

A HYPOTHESIS REGARDING THE PIVOTAL ROLE OF *miR-34a* IN AGE-RELATED SUSCEPTIBILITY TO OXIDATIVE STRESS BY TARGETING THE SIRT1/p66shc PATHWAY

As described above, previous relevant studies and our preliminary experiments have led us to hypothesize that *miR-34a* may play important roles in age-related susceptibility to oxidative stress.

Throughout life, the RPE cells are constantly challenged by high oxygen tension and exposure to photic stress, particularly in the macular region. As shown in **Figure 4**, the expression of the pro-apoptotic gene *p53* and *miR-34a* in young RPE cells was relatively low. The low expression of *miR-34a* reduces its inhibitory effect on the downstream target gene *SIRT1*, which can target the promoter regions of *p66shc* and *p53*, resulting in decreased acetylation of histone H3 bound to these regions. The

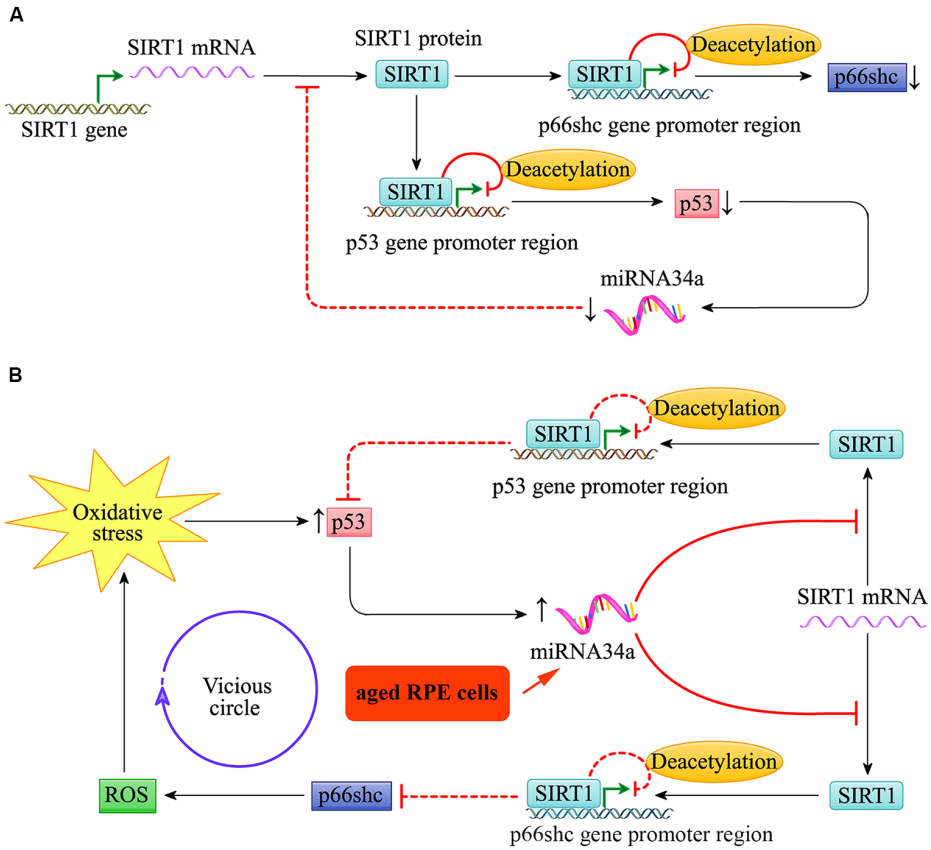

FIGURE 4 | *miR-34a* regulated the SIRT1/p66shc pathway in young and aged retinal pigment epithelium (RPE) cells. **(A)** Schematic diagram for *miR-34a* and the SIRT1/p66shc pathway in young RPE cells. **(B)** Schematic diagram for *miR-34a* and the SIRT1/p66shc pathway in aged RPE cells.

transcription of *p66shc* and *p53* is therefore inhibited. Thus, the activities of p66shc and p53 in young RPE cells are relatively low, and the cells appear to be resilient to oxidative stress to a certain extent.

Additionally, as shown in **Figure 4**, the expression of *miR-34a* in the RPE increases with cell aging, and its effect on repression of *SIRT1* translation is enhanced. Then, the SIRT1/p66shc pathway is suppressed, and histone H3 deacetylation of p66Shc and p53 by SIRT1 is inhibited. The upregulation of p66shc induces oxidative stress by restoring excessive ROS and further induces the upregulation of *miR-34a via* the transcription factor p53. This results in a vicious circle of amplification for oxidative stress and leads to reduced resistance to oxidative stress in aged RPE cells.

CONCLUSION

miR-34a may be involved in regulating susceptibility to oxidative stress by targeting the SIRT1/p66shc pathway during senescence in the RPE and the development of AMD. Targeting the inhibition of *miR-34a* may have implications in gene therapy for the early prevention and treatment of AMD. To test this hypothesis, further studies in mice with *miR-34a* knockout or overexpression are needed.

ETHICS STATEMENT

The study was approved by Ethic Committee of Shanghai Jiaotong University Affiliated Shanghai First People's Hospital, and patients provided written informed consent for participation in the study.

AUTHOR CONTRIBUTIONS

XW and NT conceived and designed the study. NT, RJ and ZZ performed the experiments and reviewed the literatures. NT and RJ wrote the article. XW, NT, RJ and ZZ reviewed and edited the manuscript. All authors read and approved the manuscript.

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frontiers in Aging Neuroscience

REVIEW
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The Post-amyloid Era in Alzheimer's Disease: Trust Your Gut Feeling

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The amyloid hypothesis, the assumption that beta-amyloid toxicity is the primary cause of neuronal and synaptic loss, has been the mainstream research concept in Alzheimer's disease for the past two decades. Currently, this model is quietly being replaced by a more holistic, "systemic disease" paradigm which, like the aging process, affects multiple body tissues and organs, including the gut microbiota. It is well-established that inflammation is a hallmark of cellular senescence; however, the infection-senescence link has been less explored. Microbiota-induced senescence is a gradually emerging concept promoted by the discovery of pathogens and their products in Alzheimer's disease brains associated with senescent neurons, glia, and endothelial cells. Infectious agents have previously been associated with Alzheimer's disease, but the cause vs. effect issue could not be resolved. A recent study may have settled this debate as it shows that gingipain, a *Porphyromonas gingivalis* toxin, can be detected not only in Alzheimer's disease but also in the brains of older individuals deceased prior to developing the illness. In this review, we take the position that gut and other microbes from the body periphery reach the brain by triggering intestinal and blood-brain barrier senescence and disruption. We also surmise that novel Alzheimer's disease findings, including neuronal somatic mosaicism, iron dyshomeostasis, aggressive glial phenotypes, and loss of aerobic glycolysis, can be explained by the infection-senescence model. In addition, we discuss potential cellular senescence targets and therapeutic strategies, including iron chelators, inflammasome inhibitors, senolytic antibiotics, mitophagy inducers, and epigenetic metabolic reprograming.

Keywords: microbiome, amyloid hypothesis, infection, senescence, inflammation

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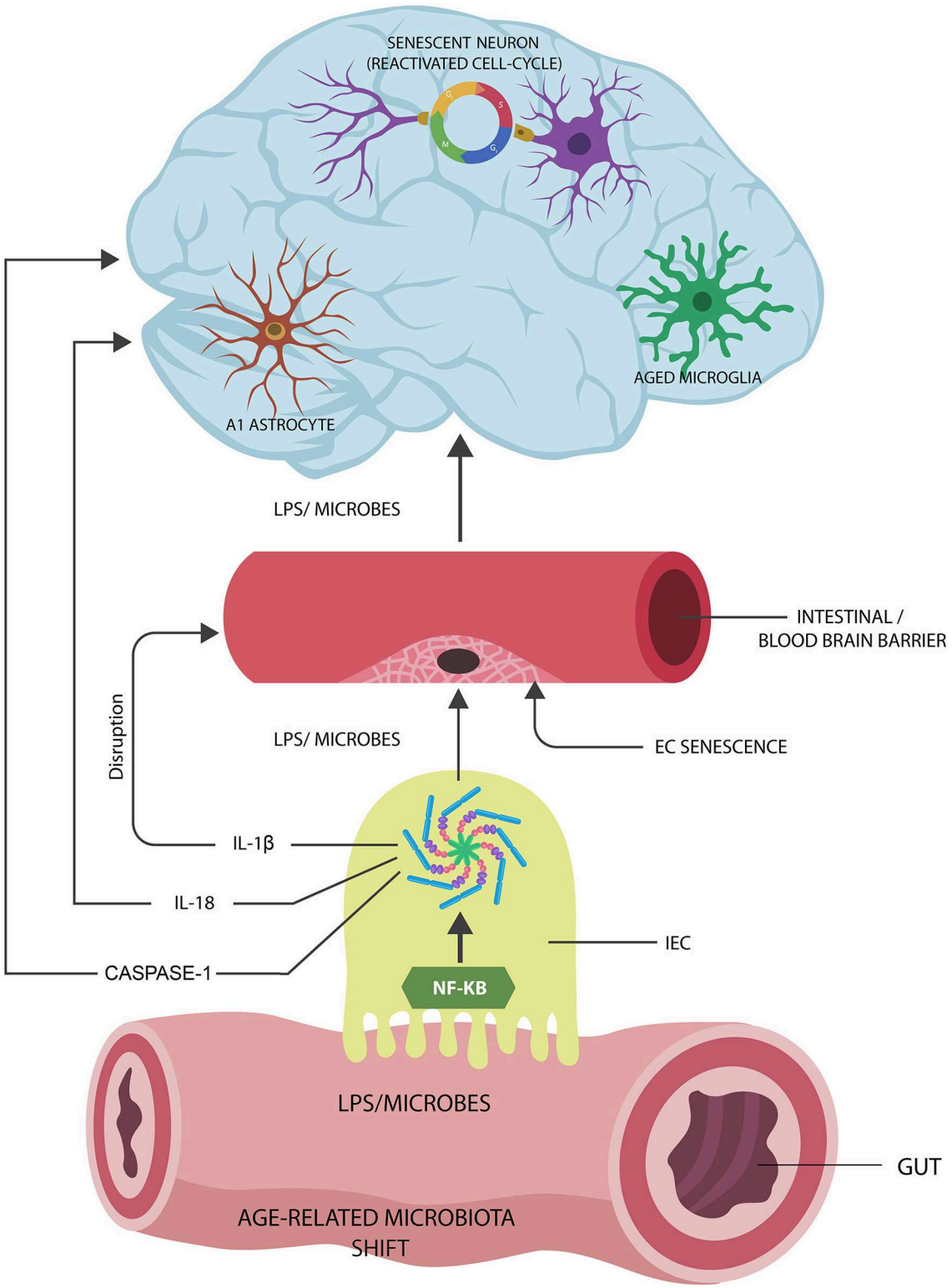
INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting an estimated 5.5 million people in the US alone (Mayeux and Stern, 2012). Advanced age is a major AD risk factor; therefore, understanding cellular senescence and its impact on endothelial cells (ECs), neurons, glia, and immune cells is an essential prerequisite for elucidating the pathogenesis of this condition (Wiseman et al., 2018).

Brain accumulation of extracellular β-amyloid and intracellular hyperphosphorylated tau are the pathological hallmarks of AD. Both neurons and astrocytes synthesize β-amyloid from amyloid precursor protein (APP), while phagocytic microglia prevent its accumulation by removing it via the triggering receptor expressed on myeloid cells-2 (TREM-2) (discussed in the section "Beta Amyloid: Friend or Foe").



Graphical Abstract | Proposed Alzheimer's disease (AD) pathogenesis: (1) Age-related gut microbiota shift leads to the upregulation of inflammagenic, lipopolysaccharide (LPS)-shedding microbial species. (2) These microorganisms activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasomes in intestinal epithelial cells (IECs), generating interleukin-1β (IL-1β), IL-18, and caspase-1. (3) IL-1β increases the permeability of intestinal and blood-brain barrier, allowing pathogen translocation into the body tissues and organs, including the brain. (4) Microorganisms and LPS induce cellular senescence in neurons, microglia, and astrocyte AD pathology.

Aging has been associated with pathological changes in microglia and astrocytes, including loss of neurotrophic properties and gain of toxic functions. These age-related glial alterations may contribute to AD pathology, marked by neuronal loss and memory impairment (discussed at length in the section "Senescent Astrocytes and Microglia").

The amyloid hypothesis postulates that accumulation and deposition of β-amyloid are the primary causes of AD, which promotes tau aggregation into neurofibrillary tangles (NFTs), ultimately triggering neuronal death (Hardy and Allsop, 1991; Wildsmith et al., 2013). Although never universally accepted, the amyloid hypothesis drove AD research for at least two decades. Lately, however, many researchers and clinicians have questioned this model as amyloid removal failed to improve memory in numerous clinical trials (Fülöp et al., 2018a). With the same token, neuroimaging studies detected significant β-amyloid deposits in 20–30% of healthy older individuals, while in many AD patients, this marker was not observed (Edison et al., 2007; Li et al., 2008; Rodrigue et al., 2013; Higashi et al., 2018). Moreover, β-amyloid was recently characterized as an antimicrobial peptide (AMP), and its accumulation in AD brains may be a reflection of increased microbial burden (Alonso et al., 2018; Fülöp et al., 2018a). AMPs are defensive biomolecules secreted by the innate immune system, including microglia and astrocytes, in response to a variety of microorganisms and malignant cells (Alonso et al., 2018). The β-amyloid-AMP connection is further supported by the observation that central nervous system (CNS) infections were diagnosed in some clinical trials, following the administration of anti-amyloid vaccines (Orgogozo et al., 2003; Brothers et al., 2018; Zhan et al., 2018).

Recent studies have reported co-localization of microorganisms with senescent neurons and glial cells in the brains of both AD patients and healthy older individuals, reviving the infectious hypothesis entertained by Alois Alzheimer himself (De Chiara et al., 2012; Bester et al., 2015; Itzhaki et al., 2016; Alonso et al., 2018; Fulop et al., 2018b; Kritsilis et al., 2018).

CNS infectious agents have been detected previously in AD patients; however, it was difficult to assess if they represented the cause or effect of this condition (Hill et al., 2014). A recent study may have settled this issue as it detected gingipain, a *Porphyromonas gingivalis* antigen, linked to AD, in the brains of healthy older persons, suggesting that they would have developed the disease if they lived longer (Dominy et al., 2019). As *P. gingivalis* is a major cause of gum disease and a modifiable AD risk factor, treatment of periodontal infection must be considered a clinical priority.

A new study identified the disruption of the blood-brain barrier (BBB) as an early aging and AD marker, suggesting a portal for microbial brain entry (Montagne et al., 2015; Nation et al., 2019). Moreover, in stroke, microorganisms were shown to directly induce EC senescence and BBB disruption, carving an entry route into the CNS (Muller et al., 2009; Saito et al., 2010; Yamazaki et al., 2016; Aguilera et al., 2018).

Aside from how microbes enter the brain, identifying their source is essential for the development of new treatments. Recent studies have demonstrated elevated levels of microbes and lipopolysaccharide (LPS) in the CNS of both healthy elderly and AD patients, suggesting the gut as their point of origin (Zhao et al., 2017; Kowalski and Mulak, 2019). Interestingly, the gut microbial shift in older individuals is characterized by the increased preponderance of Gram-negative LPS-generating microbes, pointing to the gastrointestinal (GI) tract as the potential source of brain pathogens (Kobayashi et al., 2013; Sato S. et al., 2014; Greiner and Bäckhed, 2016; Odamaki et al., 2016; Yamazaki et al., 2016; Lebrun et al., 2017; Ke et al., 2018). Furthermore, loss of immune tolerance to commensal flora in older individuals and intestinal barrier disruption suggest the gut as the likely reservoir of brain LPS and microbes (Nagpal et al., 2018) (discussed in "The Senescent Intestinal Barrier").

At the molecular level, cellular senescence has been associated with the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasomes (Yamazaki et al., 2016; Zhang W. et al., 2017; Burton and Stolzing, 2018). NLRP3 end products IL-18 and caspase-1 are associated with AD pathogenesis, while interleukin-1β (IL-1β) is an established disruptor of the BBB, linking it to microbial brain access (discussed in detail in "Senescence and Inflammasomes" section). In addition, activated NLRP3 inhibits autophagy and mitophagy (selective mitochondrial autophagy), contributing to inflammaging as the accumulation of senescent cells and damaged organelles triggers inflammation (Argaw et al., 2006; Bossù et al., 2010; Sutinen et al., 2012; Wang et al., 2014; Kim et al., 2016). Conversely, mitophagy enhancers deactivate NLRP3, limiting both cellular senescence and AD pathology (Gurung et al., 2014).

Microbiota-induced brain cells' senescence may explain other novel AD findings, including age-related neuronal genomic variation, aneuploidy, or somatic mosaicism (Argaw et al., 2006; Bossù et al., 2010). Senescent neurons reentering the cell cycle, a hallmark of AD, may account for this phenomenon, especially when apoptosis is inactivated (Paquola et al., 2016; McConnell et al., 2017; Sharma et al., 2017; Bai, 2018; Verheijen et al., 2018) (discussed in "Senescent Neurons and the Cell Cycle" section).

Senescent glial cells, probably including A1 astrocytes, have been associated with AD as they display neurotoxic functions, engaging in the elimination of viable neurons and synapses (Neher et al., 2012; Koellhoffer et al., 2017; Liddelow et al., 2017; Morizawa et al., 2017; Soreq et al., 2017; Boisvert et al., 2018; Bussian et al., 2018; Clarke et al., 2018; Forloni and Balducci, 2018; Jung and Chung, 2018). In contrast, senolysis, elimination of aggressive glia, was associated with enhanced memory in animal models, suggesting a therapeutic strategy (Koellhoffer et al., 2017; Bussian et al., 2018; Forloni and Balducci, 2018).

The infection-senescence link cannot be considered without mentioning the role of iron, a biometal indispensable to both the host and invading pathogens. Iron is well-known for inducing DNA damage and senescence in many cell types, including the ECs, linking it to microbial brain entry (Mollet et al., 2016). The association of AD with iron dysmetabolism is well-documented as, aside from microbial survival, this biometal was linked to tau pathology, reactive oxygen species (ROS), and

TABLE 1 | Perceived inconsistencies in the amyloid cascade hypothesis emphasized by novel studies.

Findings	References
β-amyloid volume does not corelate well with the degree of memory loss.	Ingelsson et al., 2004; Huber et al., 2017; Kametani and Hasegawa, 2018
NFTs, neuronal loss and memory deficit have not been observed in BRI2-Aβ AD mouse model despite abundant β-amyloid deposits.	Kim et al., 2013
Neuroimaging studies visualized significant β-amyloid deposits in up to a third of elderly individuals with no memory loss.	Edison et al., 2007; Li et al., 2008; Rodrigue et al., 2013; Higashi et al., 2018
CNS infections were observed in some clinical trials after β-amyloid clearance.	Orgogozo et al., 2003; Alonso et al., 2018; Brothers et al., 2018

neuroinflammation (Nakamura et al., 2016; Masaldan et al., 2018; Rao and Adlard, 2018).

Finally, aside from the pathogenetic mechanisms, this article discusses potential AD targets and therapeutic strategies, including inflammasome inhibitors, iron chelators, senolytic antibiotics, mitophagy inducers, and epigenetic reprograming of metabolism.

BETA AMYLOID: FRIEND OR FOE?

Amyloid cascade hypothesis, the stipulation that toxic β-amyloid oligomers and fibrils are the primary cause of AD, has been the leading paradigm that drove research in this neurodegenerative disorder for the past three decades. According to this model, β-amyloid induces the formation of NFTs, leading to neuronal and synaptic loss that ultimately impact the memory (Hardy and Higgins, 1992; Morris et al., 2014). Lately, new hypotheses have emerged as numerous anti-amyloid drugs and vaccines failed to improve cognition in clinical trials, and several studies pointed to inconsistencies in the amyloid paradigm (**Table 1**).

Recent studies have indicated that β-amyloid may function as an AMP released by the host innate immunity in response to invading pathogens (Spitzer et al., 2016; Fülöp et al., 2018a; Gosztyla et al., 2018). This is further supported by the observation that in CNS infections, microglia, and astrocytes secrete a multitude of AMP peptides demonstrated to augment host defenses (Ransohoff and Brown, 2012; Williams et al., 2012; Frost and Li, 2017). Moreover, β-amyloid, released by astrocytes and neurons, presents with antibacterial, fungicidal, and anti-herpes simplex virus, type 1 (HSV1) properties (Lukiw et al., 2010; Bourgade et al., 2016; Frost and Li, 2017; Eimer et al., 2018). This is significant since HSV1, an established disruptor of biological barriers, was found to play a major role in the etiology of both AD and intestinal pathology (Brun et al., 2018; Hogestyn et al., 2018; Itzhaki and Lathe, 2018). Interestingly, a novel study has reported that β-amyloid may work in tandem with a second AMP, probably to augment its microbicidal functions (De Lorenzi et al., 2017). Furthermore, under normal circumstances, β-amyloid may act as an opsonin, attaching to CNS microorganisms and/or their molecules to prepare them for microglial phagocytosis (**Figure 1**).

Oral and Microbial Tolerance

Other microbial antigens, including bacterial amyloids (also known as curli fibers) and *P. gingivalis*-released gingipains, may trigger β-amyloid upregulation to opsonize these "danger" molecules (Tükel et al., 2009; Hill et al., 2014; Kumar et al., 2016).

Novel studies have shown that curli fibers derived from gut microbes play a major role in promoting immune tolerance to commensals as well as oral tolerance (immune unresponsiveness to antigens administered by mouth, including food) (Barnhart and Chapman, 2006; Oppong et al., 2015). These curli functions are protective when the microbes are confined to the GI tract but become detrimental after translocation as the systemic immunity (which is not subject to oral tolerance) is activated by curli. Indeed, curli fibers were demonstrated to activate or inhibit innate immune responses, depending on the portal of entry: systemic administration of curli augments, while oral ingestion lowers immune responses (Tursi and Tükel, 2018). As curli fibers promote oral tolerance, their administration by mouth was found to restore the integrity of intestinal barrier, suggesting a potential antitranslocation strategy (Tursi and Tükel, 2018). Indeed, a bioengineered curli was recently utilized as a restorative therapy for intestinal barrier (Duraj-Thatte et al., 2018). Furthermore, like curli, oral administration of LPS derived from *Bacteroides vulgatus* and *Bacteroides dorei* was demonstrated to promote tolerance by blocking rather than activating intestinal toll-like receptor 4 (TLR-4), pointing to a mechanism for tolerization (d'Hennezel et al., 2017). Interestingly, live *B. vulgatus* and *B. dorei* were recently investigated as therapy for coronary artery disease (CAD), another condition linked to the translocation of gut microbes (Yoshida et al., 2018).

Upon accessing the CNS, curli fibers likely trigger β-amyloid synthesis, an innate immune response, causing the accumulation of this peptide. Others have suggested that curli serve as templates for β-amyloid seeding, resulting in wider CNS depositions (Friedland and Chapman, 2017). We propose that bacterial amyloids are antigens that trigger a defensive response, β-amyloid overproduction, to eliminate "danger" signals.

Aging disrupts both oral and microbial tolerance, leading to immunogenicity and inflammation in response to commensals, disrupting the intestinal barrier, a portal for microbial dissemination (Kato et al., 2003; Santiago et al., 2011).

Taken together, commensal gut microbes live in symbiosis with the host for as long as they are confined to the GI tract where the local immune system maintains a tolerant environment. This symbiosis is dramatically altered upon microorganisms' translocation as gut microbes and their antigens activate systemic immunity. Aging alters both oral and microbial tolerance, disrupting intestinal barrier and enabling microbial translocation. Upon CNS entry, microbes and their molecules induce β-amyloid overproduction. In summary, microbial containment inside the gut lumen is a key objective in the prevention of neurodegeneration, including AD.

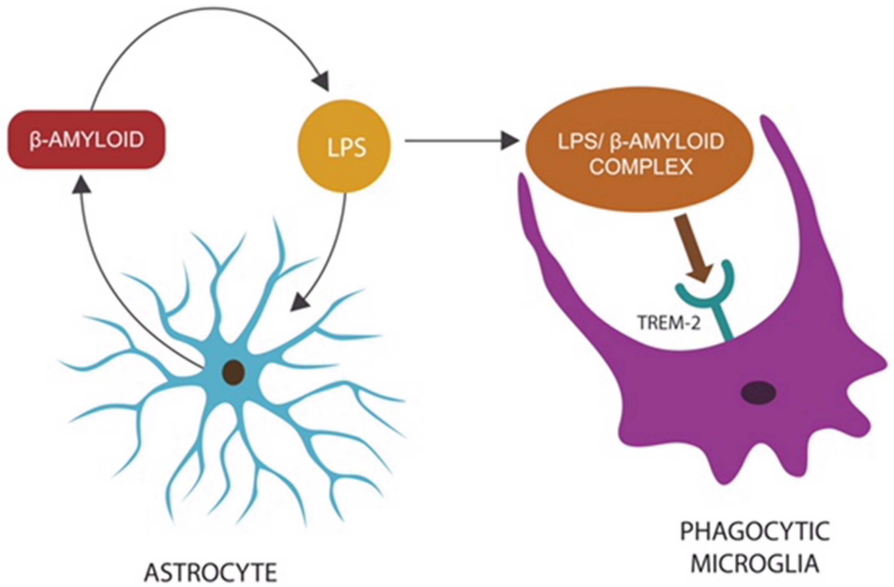


FIGURE 1 | Microbes or LPS that access the CNS comprise "danger" signals, triggering an innate immune response: the release of β-amyloid by astrocytes to opsonize "the intruder," preparing it for phagocytosis (Zhan et al., 2018). The LPS–β-amyloid complex is subsequently engulfed by microglia, eliminating the "danger." Microglial TREM-2, a β-amyloid receptor, initiates phagocytosis by binding the entire complex (Zhao et al., 2018). This mechanism may explain the reason TREM-2 genetic variants (with loss of function) present with impaired phagocytosis and β-amyloid accumulation (Guerreiro et al., 2012; Zhan et al., 2018).

Antimicrobial Peptides, Aging, and β-Amyloid

Over the past two decades, AD studies have focused primarily on the detrimental functions of β-amyloid, placing less emphasis on its physiological roles: protection against infections and cancer, BBB repair, and synaptic maintenance (Brothers et al., 2018). The presence of β-amyloid in various tissues and organs of older individuals and AD patients has gained a new significance in the light of this biomolecule functioning as an AMP (Joachim et al., 1989). For example, novel studies have detected microorganisms in older individuals' tissues, including the liver, skeletal muscles, and brain, suggesting that increased microbial burden triggers higher β-amyloid synthesis (Lluch et al., 2015). Furthermore, preclinical studies have reported age-related upregulation of AMPs in senescent tissues, implying that these defense peptides may be directly proportional to the bacterial load (Dinakaran et al., 2014). Interestingly, numerous studies over the past decade linked tissue pathogens to chronic illnesses, including CAD, cancer, stroke, type 2 diabetes mellitus (T2DM), and AD, likely implicating the translocated gut microbes in their etiology (Elkind et al., 2009; Dapito et al., 2012; Sato J. et al., 2014).

Other studies have found that the function of AMPs as antiviral and anticancer agents, suggesting that carcinogenesis and infection are handled in a similar fashion by the immune system (Hoskin and Ramamoorthy, 2007; Suttmann et al., 2008; Pandey et al., 2016). Interestingly, β-amyloid has been shown to display not only anti-HSV1 but also antimalignant properties, further suggesting an adaptive role for this peptide (Bourgade et al., 2015; Mizejewski, 2017).

AMPs have been linked to autophagy, a process involved not only in the clearance of damaged cells and molecules but also in antimicrobial defenses, as they are effective against facultative intracellular pathogens, like *P. gingivalis* (Muciño et al., 2016).

Another AMP, neuropeptide-like protein 29 (NLP-29), was found to promote the autophagy of damaged dendrites (dendrophagy) in *Caenorhabditis elegans*, extending the role of AMPs beyond infection and cancer (Lezi et al., 2018). Interestingly, fungi were shown to subvert NLP-29, inducing neuronal senescence, linking them to brain aging (Alonso et al., 2018). This is significant since fungal infections have previously been associated with AD and aging.

Other studies have reported the existence of antiretroviral AMPs, which, like antiretroviral drugs, interfere with the expression of retroviral genes, including Arc (Tencza et al., 1997; Nelson et al., 2003; Kriesel et al., 2017). Cognition-related neuronal gene Arc was demonstrated to migrate from neuron to neuron in a retroviral fashion, possibly linking antiretroviral drugs to cognition (Ashley et al., 2018; Pastuzyn et al., 2018). Interestingly, HSV1 was associated with altered transcription of Arc, linking this virus once again to neuronal senescence and memory loss (Penner et al., 2010; Bi et al., 2018; Acuña-Hinrichsen et al., 2019; Man et al., 2019). This finding is in line with novel epidemiological studies that have connected HSV1 to cellular senescence and AD (Dowd et al., 2017).

Finally, AMPs were found crucial for the integrity of intestinal barrier, suggesting their upregulation as a strategy against bacterial translocation (Robinson et al., 2015). Indeed, a recently synthesized AMP has been shown to neutralize LPS, indicating

potential antitranslocation benefits (Li L. H. et al., 2017). In addition, lactoferrin, a recently identified AMP, was found protective of intestinal barrier (Hering et al., 2017).

Senescence and Extracellular Vesicles

Most cells in the human body release extracellular vesicles (EVs) to mediate cellular crosstalk and the exchange of metabolites. Gram-negative microbes also signal with EVs (also called outer membrane vesicles) to facilitate immune evasion (Rodrigues et al., 2018). For example, *P. gingivalis* emits EVs that trigger pyroptosis in macrophages and microglia, effectively eliminating these key host defenses (Fleetwood et al., 2017). *P. gingivalis*-derived EVs have been demonstrated to contain antigens, including gingipains and fimbriae, known for disrupting ECs, causing BBB and intestinal barrier damage (Mantri et al., 2014).

Along these lines, novel studies show that the age-related gut microbial shift may be orchestrated via EVs released by microorganisms to alter local immunity and the intestinal barrier (Ahmadi Badi et al., 2017). Other studies have shown that under normal circumstances, the thymus gland releases EVs that act on gut-associated lymphoid tissue (GALT), promoting immunological tolerance to gut microbes (Skogberg et al., 2015). Age-related thymic involution may lower commensals' tolerance, engendering inflammation, and intestinal barrier disruption (Skogberg et al., 2015; Li P. et al., 2016). Interestingly, a recent preclinical study has shown that thymic EVs derived from young donors reversed the inflammaging in older recipients, suggesting that functional restoration of this gland may comprise a senotherapeutic strategy (Wang et al., 2018).

Recent studies have shown that senescent cells release more EVs than their younger counterparts, suggesting a mechanism for molecular waste disposal (Falsone and Falsone, 2015; Takasugi, 2018). For example, senescence-associated secretory phenotype (SASP) has been linked to the accumulation of cytosolic DNA in senescent cells, while DNA export via EVs was shown to inhibit this phenotype (Takahashi et al., 2018). These findings suggest that facilitation of DNA egress from senescent cells may comprise an effective senotherapeutic intervention. In this regard, the antibiotic ciprofloxacin was shown to facilitate DNA export from senescent cells, suggesting anti-SASP properties (Németh et al., 2017). Interestingly, malignant cells also display enhanced DNA export via EVs, suggesting that SASP may be associated with carcinogenesis (Rajagopal and Harikumar, 2018). Conversely, heparin was shown to block recipient cells' uptake of tumor and non-tumor-derived EVs, suggesting a potential strategy (Atai et al., 2013). Indeed, heparin was demonstrated to mimic extracellular DNA, probably interfering with SASP signaling (Jung et al., 2015; Mishra and Horswill, 2017).

Aging and Biological Barriers in Alzheimer's Disease

Cellular senescence is a program of permanent replication arrest which, under normal circumstances, lowers the risk of carcinogenesis. Prompt removal of senescent cells by the immune system prevents their accumulation and the subsequent inflammation (Oppong et al., 2015). Aging has been shown to alter this process, engendering both inflammaging and immunosenescence (Olivieri et al., 2018).

More than five decades ago, Hayflick established that cells divide a limited number of times after which they undergo replicative senescence and apoptosis (Hayflick and Moorhead, 1961). Later on, it was established that the senescence program can be activated prematurely by numerous endogenous or exogenous toxins, including the microbes and their antigens, such as LPS (Nakamura et al., 2016; Calvani et al., 2018; Kritsilis et al., 2018).

Compelling evidence indicates that cellular senescence contributes to organismal aging and the risk of developing age-related diseases, including AD (Jeyapalan and Sedivy, 2008). A growing number of studies have demonstrated that senescent cells' SASP secretome can activate the senescence program in healthy cells, propagating this phenotype throughout the surrounding tissues (Nelson et al., 2012). Conversely, senolysis, senescent cell removal, has been shown to restore homeostasis, ameliorating age-related symptoms (Baar et al., 2017; Kirkland et al., 2017). Accumulation of senescent cells and SASP-derived molecules, due to overproduction or impaired clearance, comprises an early sign of AD (Boccardi et al., 2015; Childs et al., 2015; Kritsilis et al., 2018).

Histologically, senescent cells are enlarged, presenting with β-galactosidase and lipofuscin aggregates. Functionally, they are resistant to apoptosis and metabolically active as evidenced by the intact mammalian target of rapamycin (mTOR) and the SASP secretome. Since senescent cells continue to express mTOR, targeting this molecule may comprise a senotherapeutic strategy for SASP inhibition (Walters and Cox, 2018). For example, rapamycin, an mTOR inhibitor and a natural macrolide antibiotic, was shown to block both cellular senescence and SASP (Wang R. et al., 2017; Wang S. et al., 2017). In addition, as mTOR signaling also modulates ECs synthesis of nitric oxide (NO), a trophic molecule for endothelia, targeting mTOR may restore the integrity of biological barriers, including the BBB (Cheng et al., 2008; Van Skike and Galvan, 2018). Interestingly, *P. gingivalis* was found to alter mTOR signaling, linking this microbe once again to EC senescence (Stafford et al., 2013). Conversely, azithromycin, an anti-*P. gingivalis* macrolide antibiotic and mTOR modulator, was found to have senotherapeutic properties, indicating potential benefits in AD (Maezono et al., 2011; Ratzinger et al., 2014; Ozsvari et al., 2018; Weng et al., 2019).

The Senescent Intestinal Barrier

The gut microbial community, consisting of bacteria, fungi, archaea, viruses, and protozoans, live in symbiosis with the human host, contributing to metabolism and immune homeostasis in exchange for nutrients and habitat (Jandhyala et al., 2015). Intestinal epithelial cells (IECs), a one cell layer, separate the host from trillions of microbes and antigens, preventing their translocation outside of the GI tract where systemic immunity is intolerant of them. Aside from IECs, GALT (the GI tract immune system) contributes to the integrity of the intestinal barrier by blocking immunogenicity to beneficial

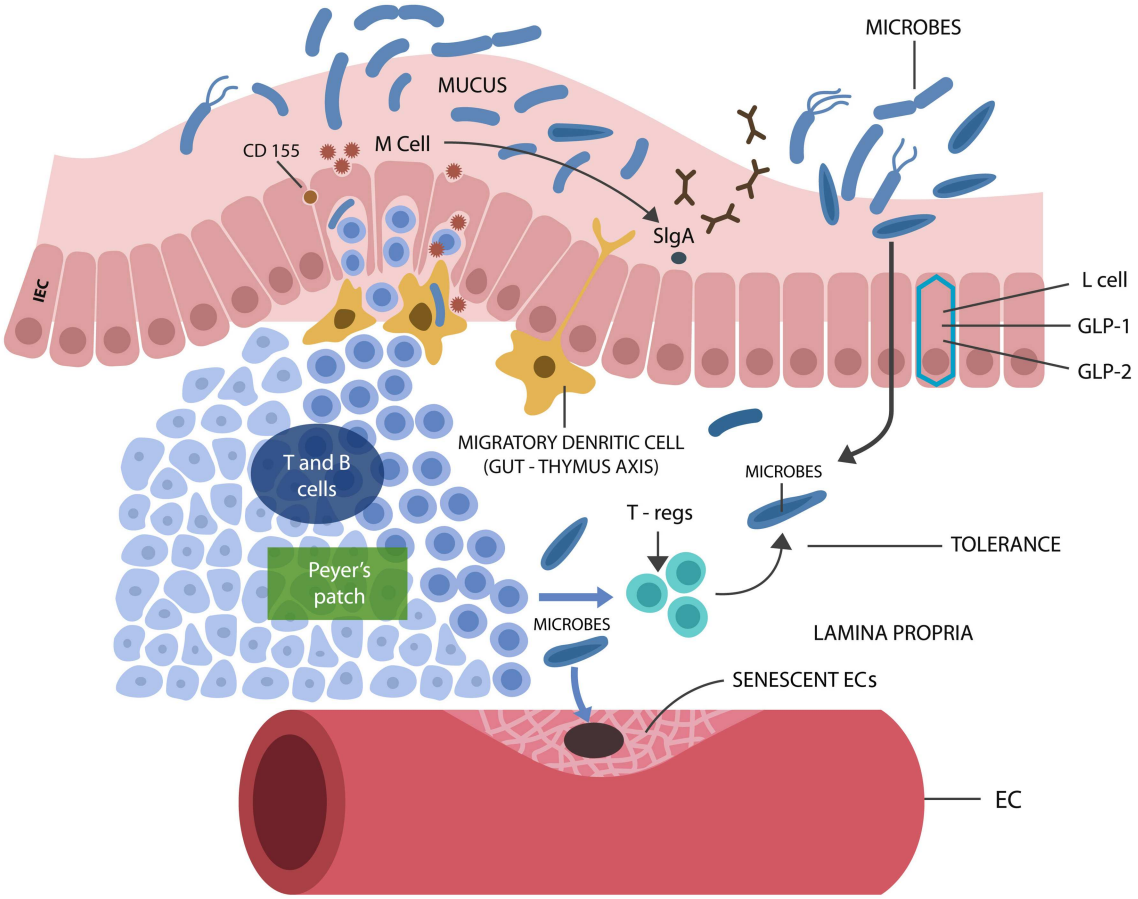


FIGURE 2 | M cells interact with luminal microbes, introducing microbial antigens to the T and B cells, engendering tolerance via Tregs and IL-10 secretion. M cells promote the expression of the mucosal protector, SIgA. L cells synthesize IECs protecting biomolecules GLP-1 and GLP-2. Senescent ECs cause endothelial disruption, allowing pathogens into the circulatory system, from where they find their way into the CNS.

microorganisms, ensuring their containment in the GI tract (Hwang et al., 2012). Loss of tolerance to gut commensals was shown to cause immune activation, barrier disruption, and translocation (Ramanan and Cadwell, 2016). GALT facilitates microbial tolerance by promoting the differentiation of IL-10 secreting B and regulatory T cells (Tregs) (Kelsall and Leon, 2005) (**Figure 2**). In addition, GI tract lactobacilli, bifidobacteria, and *Bacteroides* also facilitate microbial acceptance as they promote oral and microbial tolerance (Cebula et al., 2013; Kayama and Takeda, 2014; Nakamoto et al., 2017). Tolerance is believed to be initiated during the early development when GALT receives thymic input, generating a long-lived population of T cells that facilitate microbial tolerance even after the involution of this gland (Cebula et al., 2013).

Maintenance of a microbe-friendly GI tract milieu, completely isolated from the systemic immunity, is crucial for averting microbial translocation, a phenomenon that may initiate age-related diseases, including AD. Conversely, restoring the integrity of biological barriers and limiting microbial translocation should be the primary objective of senotherapy. Indeed, recent studies have associated mTOR inhibition with the restoration of intestinal barrier damaged by *P. gingivalis* (Xu et al., 2015; Nakamoto et al., 2017; Ji et al., 2018; Kato et al., 2018). Inhibitors of mTOR receptors were shown to lower GI tract immunogenicity as a bilateral regulation exists between gut microbes and mTOR, in which the later regulates microbial composition, while the former modulates mTOR expression (Salem et al., 2018). This is significant as it demonstrates that mTOR manipulation may reverse the age-related shift in gut microbiota, lowering the preponderance of pathogenic species and preserving intestinal barrier. On the other hand, microbiota manipulation, for example, via the fecal transplant, may reverse the preponderance of gut LPS-generating species in favor of beneficial microbes, such as *Bacteroides*, protecting intestinal barrier (Nagpal et al., 2018). Along these lines, a large epidemiological study found that *Bacteroides* species were less represented in the GI tract of AD patients compared to other microbes, indicating that microbiota manipulation may preempt neurodegeneration (Saji et al., 2019).

Age-related microorganismal shift toward Gram-negative bacteria and LPS induces EC senescence and apoptosis and IEC and GALT damage, disrupting the intestinal barrier (Hoyt

et al., 1996; Richter et al., 2012; Nagele et al., 2013; Ke et al., 2018; Sanada et al., 2018; Hou et al., 2019). In addition, several gut microbes were demonstrated to upregulate the host tumor necrotic factor alpha (TNF-α) and interferon-gamma (IFN-γ), increasing intestinal permeability (Al-Sadi and Ma, 2007). Furthermore, these cytokines activate NLRP3, generating IL-1β, a known BBB disruptor (Wang et al., 2014).

Does Aging Start in the Gut?

It was recently reported that GALT dysfunction may occur prior to the systemic immune deterioration, suggesting that immune aging and, perhaps, aging in general could originate in the GI tract with the loss of tolerance to commensals and barrier disruption (Koga et al., 2000; Sato S. et al., 2014).

It was recently demonstrated that glucagon-like peptide-1 (GLP-1) secreted by the gut enteroendocrine (L) cells binds to its receptor, GLP-1R, facilitating immunological tolerance (Yusta et al., 2015). Others have shown that under normal circumstances, LPS upregulates GLP-1, suggesting that this hormone may display AMP-like characteristics (Lebrun et al., 2017). Interestingly, GLP-1R agonists were recently demonstrated to block the conversion of trophic into A1 astrocytes, linking this peptide to CNS homeostasis (Yun et al., 2018). GLP-1R agonists, established therapeutics for T2DM, were previously shown to protect cognition; thus, liraglutide and exenatide are currently in clinical trials for AD and Parkinson's disease (PD), respectively (Kim et al., 2017; Batista et al., 2018; Cummings et al., 2018).

Aside from GLP-1 secretion, L cells sense pathogen-derived molecules, likely suggesting that GLP-1 functions as an AMP (Greiner and Bäckhed, 2016; Lebrun et al., 2017). Aging has been associated with decreased number of L cells, accounting for the loss of GI tract immunological tolerance (Drozdowski and Thomson, 2006; Wu et al., 2018). Aside from L cells, GALT dysfunction may be linked to the loss of membranous (M) cells, known for producing secretory immunoglobulin A (SIgA), an IEC immune protector (Mantis et al., 2011; Kobayashi et al., 2013; Sato S. et al., 2014; Ohno, 2016). Furthermore, aging has been associated with the loss of LPS-binding protein (LBP), another possible AMP, known for its trophic effects on the intestinal barrier (Schmucker et al., 2003; Hamann et al., 2005; Richter et al., 2012).

Another mechanism responsible for tolerance to commensal flora may involve the CD155 poliovirus receptor, expressed by M cells. CD155 binds to T cell co-inhibitory receptor TIGIT (T cell Ig and ITIM domain), initiating the release of IL-10 (Lozano et al., 2013; Ohno, 2016). Dysfunctional TIGIT has been associated with T cell senescence, linking immune aging to the GI tract (Solomon and Garrido-Laguna, 2018). On the other hand, TIGIT blockade, a well-known cancer treatment, activates immunity (Song et al., 2018). This is significant, since T cell co-inhibitory receptors are routinely hijacked by pathogens to lower host immunity and evade detection (Attanasio and Wherry, 2016). For example, *P. gingivalis* is known for subverting programmed death-1 (PD-1), a co-inhibitory receptor, to escape host immunity (Groeger et al., 2017).

The Senescent Blood-Brain Barrier

ECs pave the interior wall of blood vessels and capillaries, contributing to blood flow, platelet function, and immunity (Ross, 2018). Microorganisms use the host circulatory system to travel around the body, crossing the ECs to enter and exit the bloodstream (Lubkin and Torres, 2016). To facilitate this process, pathogens trigger EC senescence and apoptosis, disrupting biological barriers, including the BBB (Kim, 2008). This action is counteracted by the ECs' secretion of NO, an endothelial protector (Hayashi et al., 2008; Austin et al., 2013). Decreased NO generation was associated with NLRP3 inflammasome activation, aging, and AD (Mao et al., 2013; Sverdlov et al., 2014).

Astrocytic end-feet, ECs, and pericytes comprise the BBB or neurovascular unit (NVU), which feeds neuronal networks, enabling their function (Filosa et al., 2015; Tarantini et al., 2016). Several studies have shown that BBB disruption is an early AD marker, indicating a potential portal for microbial entry into the CNS (Montagne et al., 2015; Nation et al., 2019). A novel study measured platelet-derived growth factor receptor-beta (PDGFRβ), a pericyte marker, and showed that its deficit increased the permeability of BBB, contributing to AD (Nation et al., 2019). In addition, recent AD postmortem studies have associated loss of pericytes with BBB dysfunction in various cortical areas, including the hippocampus (Miners et al., 2017; Schultz et al., 2018).

Pericytes have been reported to play a major role in CNS antimicrobial defenses by secreting microbicidal molecules, including IL-1β, IL-6, and TNF-α (Alcendor et al., 2012; Hurtado-Alvarado et al., 2014; Stark et al., 2018). Several pathogens were demonstrated to evade host immunity by subverting the pericytes, linking these cells to microbes and their portal of entry (Alcendor et al., 2012). For example, a new study has demonstrated that heme-dependent pathogens can damage ECs and pericytes to extract this iron protein from the circulating red blood cells (Choby and Skaar, 2016; Erdei et al., 2018). Along these lines, to acquire heme, *P. gingivalis* releases gingipain, which attaches to the EC receptor E-selectin, disrupting these cells (Komatsu et al., 2012; Smalley and Olczak, 2017). Other studies have reported that fimbriae, another *P. gingivalis* antigen, binds EC-expressed complement receptor 3, inducing immune tolerance to enter the CNS undetected (Hajishengallis et al., 2008). This is significant because upregulated complement component C1q and its downstream molecule C3 were linked to AD via A1 astrocytes induction (Wu et al., 2016; Liddelow et al., 2017; Morgan, 2017). ECs are extremely susceptible to microbial disruption as they express the tolerance-inducing complement pathway genes; therefore, when pathogens subvert these cells, they trigger immune unresponsiveness (Walker et al., 2007; Shi et al., 2017).

Aside from *P. gingivalis*, *Helicobacter pylori* and *Escherichia coli* were found to induce EC senescence and apoptosis, linking them to the disruption of biological barriers (Munshi et al., 2002; Krishnan et al., 2012). Moreover, HSV1, connected to both atherosclerosis and AD, was demonstrated to invade ECs, activating glycogen synthase kinase 3 beta (GSK3β), an enzyme previously associated with neurodegeneration (Key et al., 1990;

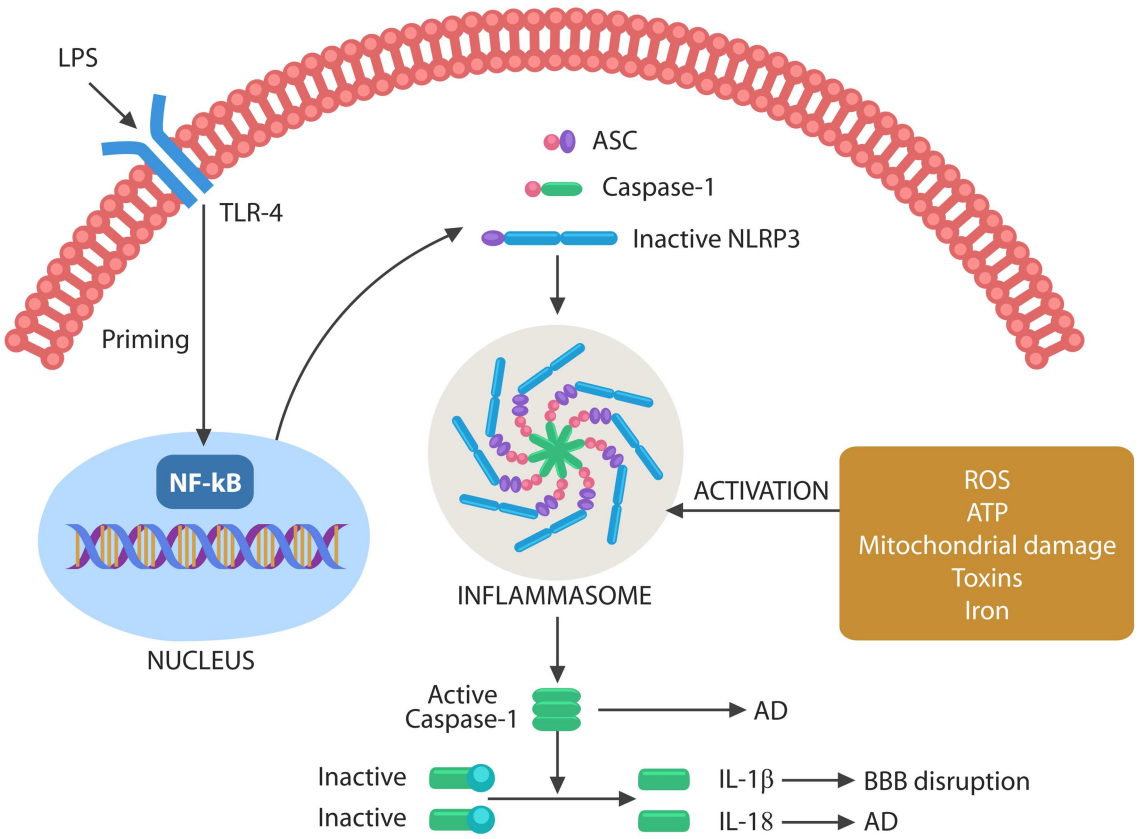


FIGURE 3 | Schematic representation of NLRP3 activation by microbes or their molecules. Microbes or LPS binds to TLR-4, activating the nuclear NF-kB that primes NLRP3. The second step necessary for NLRP3 activation can be composed of various exogenous or endogenous stimuli, including ROS, ATP, DNA, defective mitochondria, iron, and toxins. Assembled inflammasome activates caspase-1, which in turn cleaves immature IL-1β and IL-18 into their active forms. Caspase-1 and IL-18 have been involved in AD pathogenesis, while IL-1β disrupts the BBB, facilitating brain translocation of gut microbes.

Piacentini et al., 2015; Rybakowski, 2019). Interestingly, lithium, a GSK3β blocker, also presents with anti-HSV1 properties, suggesting a protective effect on endothelia (Amsterdam et al., 1990; Bosche et al., 2016). Indeed, the beneficial effect of lithium in AD may involve endothelial restoration (Bosche et al., 2016; Cummings et al., 2018).

Immune aging or immunosenescence promotion, engendering immune failure, is another mechanism utilized by gut microbes to avoid detection and access the CNS unopposed (Blazkova et al., 2009; Alvarez-Arellano and Maldonado-Bernal, 2014; Aguilera et al., 2018; Costantini et al., 2018).

Taken together, pathogen-induced pericyte and EC senescence and apoptosis along with impaired immune function facilitate microbial translocation into the brain with subsequent AD pathology.

Senescence and Inflammasomes

It has been well-established that inflammation and cellular senescence are closely related, but the role of pathogens in this process has been less emphasized (Balistreri et al., 2013; Secher et al., 2013; Lewinska and Wnuk, 2017; Rybakowski, 2019). At the molecular level, cellular senescence is believed to be initiated by the nuclear translocation of the NF-kB transcription factor, a molecular event that primes NLRP3 inflammasomes (McCool and Miyamoto, 2012; Birch and Passos, 2017) (**Figure 3**).

Several antibiotics, including minocycline and macrolides, present with both antimicrobial and anti-inflammatory properties as they de-escalate NLRP3 (Pradhan et al., 2016). Recent studies have shown that minocycline also presents with senolytic properties as aside from inhibiting NLRP3, it facilitates senescent cell removal (Labro, 2002; Li J. et al., 2016; Lee et al., 2017).

Other antibiotics with senotherapeutic actions include azithromycin and rifampicin, suggesting that infection, inflammation, and cellular senescence are related phenomena (Golegaonkar et al., 2015; Lendermon et al., 2017; Ozsvari et al., 2018).

Inflammasomes are macromolecular complexes that sense pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) via cytosolic NLRP3 composed of NOD-like receptors, adapter proteins, apoptotic speck containing molecules with a CARD (ASC), and pro-caspase-1 (Uekawa et al., 2004; Broz and Monack, 2011; Wang et al., 2014; Schetters et al., 2018).

Inflammasome assembly requires two steps, a priming event, triggered by NF-kB nuclear translocation, and an activating step, induced by toxins, iron, mitochondrial damage, cytosolic DNA, extracellular ATP, or ROS. Inflammasome assembly activates caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into their mature forms (**Figure 3**). These cytokines have been involved in both BBB disruption and AD (Heneka et al., 2013; Freeman and Ting, 2016; Malik and Kanneganti, 2017).

Pyroptosis is a programmed cell death triggered by infection-induced NLRP3 activation mediated by caspase-1,−4, and−5. Caspase-1 is the product of NLRP3 assembly, while caspase-4 and−5 are LPS activated (Man et al., 2017). Caspases perforate cell membranes via gasdermin D, a pore-forming protein, spilling intracellular content into the ECS, a process that triggers inflammation (Ma et al., 2018). Pyroptosis has been documented in the pathogenesis of neurodegenerative disorders (Walsh et al., 2014; Wang et al., 2014; Ma et al., 2018). In fact, it is believed that pyroptosis and not apoptosis leads to neuronal loss in AD (Bai, 2018; Fali et al., 2018). Moreover, recent studies have reported NLRP3-induced pyroptosis in ECs, likely explaining the disruption of intestinal barrier and BBB during the aging process (Lei et al., 2018; Zhaolin et al., 2018).

Mitochondrial damage was recently recognized as a key NLRP3 activator, emphasizing the role of these organelles in both aging and AD. For example, mitochondrial dysfunction-associated senescence (MiDAS) is an aging phenotype with a specific secretome which, like SASP, can propagate cellular aging throughout the tissues (Gallage and Gil, 2016). Other studies have shown that mitochondrial content, especially mtDNA, in contact with the cytosol activates cellular senescence and SASP, while cytosolic DNA removal inhibits both (Takahashi et al., 2018; Takasugi, 2018). *P. gingivalis*-induced cellular senescence may involve mtDNA as this microbe has been known for inflicting mitochondrial damage (Bullon et al., 2011). This is in line with a novel study that identified cell free-DNA (cfDNA) as an aging and AD marker, suggesting that cytosolic DNA exported into the ECS may suppress SASP (Takousis et al., 2018; Teo et al., 2018). Together, these studies suggest that enhancing the clearance of cytosolic DNA may facilitate senolysis, lowering the senescent cell burden in tissues and organs (Takousis et al., 2018; Teo et al., 2018).

Senescent Neuron and the Cell Cycle

Accumulating evidence indicates that aging neurons activate a special senescence program, defined as senescence after differentiation (SAD), a phenotype marked by upregulation of β-galactosidase, lipofuscin, SASP, and IL-6 (Jurk et al., 2012; Naylor et al., 2012; Tan et al., 2014). Along these lines, a new study identified senescent neurons in the orexinergic, cholinergic, and dopaminergic tracts of the brainstem and basal forebrain, probably indicating that some neuronal populations undergo senescence earlier than others (Panossian et al., 2011). As opposed to senescent somatic cells which arrest proliferation irreversibly, old neurons may do the opposite, reenter the cell cycle, triggering their own demise (Paquola et al., 2016; McConnell et al., 2017; Verheijen et al., 2018). Indeed, the expression of neuronal cell cycle proteins was detected in both healthy seniors and AD patients, suggesting that these molecules are senescence associated (van Leeuwen and Hoozemans, 2015; Frade and López-Sánchez, 2017). In addition, a novel AD postmortem study has linked neuronal senescence with both aggregated tau and neuronal cell cycle reentry, identifying both as age-related traits (Musi et al., 2018). Moreover, aberrant neuronal cell cycle reentry has been associated with a senescence-linked protein, cyclin-dependent kinase 5 (Cdk5), which phosphorylates both tau and the retinoblastoma protein (pRb) (Mao and Hinds, 2010). The nuclear localization of Cdk5 was found necessary for maintaining neuronal cells in post-mitotic state (Zhang et al., 2008). Conversely, egress of this protein from the nucleus activates the cell cycle (Hamdane et al., 2005; Crews et al., 2011; Hradek et al., 2015; Na et al., 2015). This may be the case in AD, in which microbes and/or LPS may trigger Cdk5 nuclear export and neuronal cell cycle activation (Zhang et al., 2016). In PD animal models, neuronal Cdk5 was found to activate NLRP3, initiating the cell cycle (D'Angelo et al., 2017; Bai, 2018; Wilkaniec et al., 2018). Indeed, to interact with the cytosolic NLRP3, Cdk5 must exit the nucleus, enabling this phenomenon. Conversely, LPS removal via LBP probably promotes Cdk5 nuclear reentry, stabilizing neuronal cells in post-mitotic state (Pretorius et al., 2018).

Iron is a well-known activator of neuronal cell cycle, probably due to DNA damage and NF-kB/NLRP3 activation, suggesting that iron chelators may have senotherapeutic properties (Nakamura et al., 2016; Ashraf et al., 2018; Manickam et al., 2018) (discussed in "Senescence and Iron" section).

It is currently believed that senescent post-mitotic cells, including the neurons, reenter the cell cycle to trigger their own demise. This is thought to take place as these cells lack the molecular machinery to complete mitosis, activating death programs instead (Kruman et al., 2004). For example, in muscular degeneration, adult post-mitotic myocytes were shown to reengage the cell cycle, triggering their own death (Sharma et al., 2017). Conversely, cell cycle inhibitors were recently found neuroprotective in AD organoid models, suggesting a possible therapeutic strategy (Hor et al., 2018).

Most recent studies have suggested that some neuronal populations reentering the cell cycle do not always undergo cell death but remain in the aneuploid state for the rest of their lives (Frade and López-Sánchez, 2017). For example, loss of the p53 tumor suppressor, a DNA repair protein, was associated with neuronal survival in the aneuploid state (Barrio-Alonso et al., 2018).

Neuronal cells have recently been reported to present with variable DNA content from one cell to another (somatic mosaicism), especially during the early development and old age (Paquola et al., 2016; McConnell et al., 2017; Sharma et al., 2017; Caneus et al., 2018; Leija-Salazar et al., 2018; Verheijen et al., 2018; Villela et al., 2018). This finding led to the development of a new field, defined as the brain somatic mosaicism (Paquola et al., 2016; McConnell et al., 2017). We speculate that this phenomenon is the result of senescent neurons reengaging the cell cycle and surviving in aneuploid states. In AD, neuronal somatic mosaicism may be reflected in the aneuploidy-induced APP gene variants (Bushman et al., 2015). Interestingly, a

recent study has suggested that APP variants are generated via RNA retro-insertion into the DNA, suggesting that antiretroviral drugs may be beneficial for AD (Lee et al., 2018). Others have argued that patients with HIV-associated neurocognitive disorders (HANDs) rarely experience improved memory while in treatment with antiretrovirals (McArthur et al., 2010; Vance et al., 2013). These contradictory findings indicate that more studies are needed to clarify the role of these agents in AD.

Finally, two questions beg for answers: Are aneuploid neurons viable and does it make sense to facilitate their survival?

Novel studies in regenerative medicine reported that facilitating cell cycle completion in senescent cardiomyocytes prevented their apoptosis (Anversa and Leri, 2013; Hesse et al., 2017; Locatelli et al., 2018). Helping neurons survive the cell cycle engagement may comprise a therapeutic strategy in AD, but only if aneuploid cells are functional (Frade and López-Sánchez, 2017). Conversely, preventing neurons from engaging the cell cycle, a more straightforward approach, may be accomplished by blocking the nuclear export of Cdk5 or suppressing this protein in the cytosol with Cdk5 blockers or lithium (Zhang et al., 2008; Carvalho et al., 2013).

Senescent Astrocytes and Microglia

Astrocytes are the most numerous brain cells and their end-feet, ECs and pericytes comprise the BBB. Recent studies report that astrocytes are innate immune cells that, along with microglia, play a key role in the phagocytic removal of molecular waste, dead, or dying cells (Farina et al., 2007; Ransohoff and Brown, 2012; Morizawa et al., 2017). In addition, astrocytes generate AMPs, including β-amyloid, that may opsonize pathogens, facilitating their removal (**Figure 1**).

Preclinical studies have reported that astrocytes undergo both replicative and stress-induced senescence characterized by SASP, p16INK4a, and p21CIP1 markers; however, the difference between senescent and reactive astrocytes is not entirely clear at this time (Hou et al., 2018; Kritsilis et al., 2018; Maciel-Barón et al., 2018; Perez-Nievas and Serrano-Pozo, 2018). Recent studies seem to indicate that these phenotypes may be closely related or even identical as upregulated inflammatory and synapse-eliminating genes were found in both senescent and reactive astrocytes (Crowe et al., 2016; Boisvert et al., 2018). Along these lines, the aggressive A1 astrocytes may be senescent as they also upregulate inflammatory genes and eliminate healthy synapses (Liddelow et al., 2017; Morizawa et al., 2017; Clarke et al., 2018; Vilalta and Brown, 2018). In support of this hypothesis comes the recent finding that senescence-upregulated cytokines, TNF-α and IL-1, induce the A1 phenotype (Cartier et al., 2014; Altieri et al., 2017; Liddelow et al., 2017; Li P. et al., 2017; Yun et al., 2018).

Microglia are CNS innate immune cells, which, like macrophages at the body periphery, are vigilant and motile, characteristics that help them scrutinize the brain parenchyma, searching for "danger signals." Microglia respond to invading pathogens by releasing pro-inflammatory cytokines which can trigger astrocytic senescence and reactivity (Cartier et al., 2014). In addition, under normal circumstances, microglia engulf senescent or dead cells, preventing their accumulation and the subsequent inflammation (Jung and Chung, 2018). Aging and immunosenescence were shown to alter microglial phagocytic function, generating inflammaging triggered by the accumulation of molecular waste and cellular corpses (Neumann et al., 2009; Koellhoffer et al., 2017).

Dystrophic microglia with growth arrest and senescent markers have been demonstrated in AD patients, but the difference between the reactive and dystrophic phenotype is unclear at this time (Flanary et al., 2007; Mosher and Wyss-Coray, 2014). Several studies have reported that although senescent microglia may lose their neuroprotective functions, their ability to mount inflammatory responses is preserved and even enhanced (Sierra et al., 2007; Davies et al., 2017). For example, senescent microglia have been shown to upregulate their TLRs, triggering exaggerated inflammation in response to minimal LPS stimulation. On the other hand, continuous LPS presence in the microglial environment induces immunosenescence with deficient phagocytosis (Yu et al., 2012). Recently, "dark," hypervigilant microglia have been reported, likely representing senescent cells with aberrant phagocytic function (Bisht et al., 2016). Indeed, several studies report that in the presence of LPS, senescent microglia and astrocytes became neurotoxic, engaging in the phagocytosis of healthy neurons and synapses (von Bernhardi et al., 2015; Lana et al., 2017). Moreover, preclinical studies have shown that LPS-exposed microglia promote extracellular trafficking of hyperphosphorylated tau, a phenomenon inhibited by IL-10 (Liu et al., 2016; Magalhães et al., 2017; Hopp et al., 2018; Kametani and Hasegawa, 2018). Furthermore, microglial NLRP3 and its end products, IL-18, caspase-1, and IL-1β, have been associated with cellular senescence and AD (Griffin et al., 2006; Ojala et al., 2009; Cabral and de Lima, 2017). Conversely, caspase-1 inhibition ameliorates AD symptoms in animal models, suggesting a novel target (Yu et al., 2009; Cabral and de Lima, 2017; Flores et al., 2018).

Taken together, senescent microglia, incapable of proper immunosurveillance and phagocytosis, contribute to the accumulation of molecular waste, dead or dying cells, inducing inflammaging and immunosenescence. Astrocytes may respond to these microenvironmental changes by converting to the A1 phenotype marked by aberrant elimination of healthy synapses and neurons, a possible pathogenetic mechanism of AD.

SENESCENCE AND AEROBIC GLYCOLYSIS: GOT LACTATE?

In the nineteenth century, Otto Warburg noticed that cancer cells converted glucose to lactate even in the presence of oxygen, a metabolic modality defined as aerobic glycolysis (AG). Compared with healthy cells, which oxidize glucose in the mitochondrion via oxidative phosphorylation (OXPHOS), cancer cells prefer cytosolic AG that generates excessive amounts of lactate (Potter et al., 2016). These observations beg the question: Why do cancer cells need lactate?

Recent findings helped solve this dilemma by revealing that cancer, like microorganisms, escapes detection by reprograming host immune cells to AG, a metabolic modality associated with immune tolerance (Roland et al., 2014; San-Millán and Brooks, 2016). In addition, lactate generates an acidic microenvironment which inhibits the host immune system (Romero-Garcia et al., 2016). Furthermore, lactate upregulates snail, a tumorigenic protein (encoded by the SNAI2 gene) which inhibits host cellular senescence, a key antitumor defense (Li X. et al., 2018).

Novel studies found that AG is the metabolic preference not only of cancer cells but also of many healthy tissues, including the brain (Demetrius et al., 2015; Yellen, 2018). Under normal circumstances, 10–12% of brain glucose is catabolized via AG despite oxygen availability (Goyal et al., 2017). Furthermore, the brain regions most dependent on AG are those involved in rapid activation and information processing, such as cognition, memory, and alertness (Dienel and Cruz, 2016).

Along similar lines, it was recently reported that immune cells and ECs preferentially utilize AG, especially when exposed to LPS or pathogens, suggesting that for rapidly proliferating cells, the slower OXPHOS may be an inadequate energy modality (Jones and Bianchi, 2015; Boitsova et al., 2018; Escoll and Buchrieser, 2018; Liu R. et al., 2018; Salmond, 2018). On the other hand, senescent cells rely almost exclusively on OXPHOS, indicating that loss of AG is an aging biomarker (Wen et al., 2012; Li et al., 2013; Goyal et al., 2017). The molecular mechanism of age-related AG loss is incompletely understood; however, under normal circumstances, lactate is synthesized by astrocytes, a neurotrophic function that may be lost in senescent cells (Riske et al., 2016). It has been established that lactate interacts with its receptor GPR81 (also called HCAR1) to generate rapid ATP surges required for neuronal activation (Bergersen and Gjedde, 2012; Díaz-García et al., 2017). Unlike AG, OXPHOS may be incapable of supplying the neurons with large amounts of energy on short notice (Díaz-García et al., 2017).

Aside from the CNS, lactate-GPR81 signaling plays a key role in the GI tract, where it maintains the integrity of intestinal barrier by positively regulating IL-10 (Ranganathan et al., 2018). Aging alters the lactate-GPR81 axis, disrupting both commensals tolerance and the intestinal barrier. In addition, age-related loss of gut *Lactobacillus* species, a major source of intestinal lactate, may impair GPR81 signaling, increasing intestinal permeability and facilitating microbial translocation (Walter, 2008). Moreover, as lactate-GPR81 interaction blocks the NLRP3 activation, agonists at these receptors may present with senotherapeutic properties (Hoque et al., 2014; Errea et al., 2016; Nolt et al., 2018).

In AD, due to compromised lactate-GPR81 signaling, AG may be unavailable, rendering neuronal cells totally dependent on mitochondrial OXPHOS. However, as the aging process also impairs mitochondria, OXPHOS becomes unreliable, triggering an energy crisis (Fong et al., 2016). Furthermore, the compensatory mechanisms, including mitochondrial fission, fusion, and mitophagy, are also compromised in AD, further lowering OXPHOS and deepening the crisis (Santos et al., 2010; Fang et al., 2016; Kerr et al., 2017).

Immunosenescence and Inflammaging

Immune system aging is closely linked to gut microbes and the loss of AG. Aging affects both the innate and adaptive immunity, but some cells are more affected than others (Burton and Stolzing, 2018). For example, AG-relying effector T cells are more impacted by age than the OXPHOS-preferring memory T cells (Carlos et al., 2018). As a result, antigens are remembered in old age, but they may trigger poor immune activation as evidenced by older individuals' weak response to vaccines (Lord, 2013).

Age-related immune alterations are captured by two words, inflammaging, denoting excessive innate immune activation, and immunosenescence, referring to the depletion of adaptive immune cells (Ventura et al., 2017; Fülöp et al., 2018a). The innate immune changes affect macrophages and natural killer (NK) cells at the body periphery as well as microglia and astrocytes in the CNS (Solana et al., 2018).

The NF-kB/NLRP3 axis was shown to regulate immune aging via proinflammatory cytokines IL-6, TNF-α, IL-1β, and IL-18 (Heneka et al., 2013; Couturier et al., 2016; Rea et al., 2018). Moreover, peripheral infections and inflammation were linked to microglial senescence, suggesting that interventions at the body periphery may influence central immunity (Netea and van der Meer, 2017; Cao and Zheng, 2018; Wendeln et al., 2018). Furthermore, infection with various pathogens, including cytomegalovirus (CMV), human immunodeficiency virus (HIV), HSV1, and *Toxoplasma gondii*, was implicated in immunosenescence and inflammaging, connecting these phenomena to microbes and their molecules (Solana et al., 2018). This is in line with the immune risk phenotype (IRP), a morbidity marker described in the elderly with CMV infection (Olsson et al., 2000).

Immunosenescence, marked by the depletion of adaptive immune cells, reflects thymic involution, a process starting in childhood and progressing at a rate of 3% per year throughout the adult life (Gui et al., 2012). The gradual loss of thymic function is manifested by a decrease in naïve T cells, increased number of memory cells, and downregulation of T cell receptors (TCRs) (Deleidi et al., 2015). Novel preclinical studies have linked thymic involution to the activation of the NF-κB/NLRP3 axis, while caspase-1 inhibitors were shown to restore thymic lymphopoiesis in elderly (Youm et al., 2012; Wen et al., 2018). Interestingly, viruses, bacteria, fungi, and parasites were demonstrated to infect the thymus directly, probably inducing senescence and premature atrophy (Nunes-Alves et al., 2013). This is significant as it links thymic involution to the loss of intestinal Tregs, impaired barrier function, and microbial translocation. Indeed, a thymus-gut axis was described during the early development when dendritic cells migrate from the GI tract to "educate" the thymus in commensals tolerance (Lathrop et al., 2011; Jain and Seed, 2016) **Figure 2** (also discussed in "The senescent intestinal barrier").

Age-related thymic involution was also associated with the loss of gut IL-10-secreting B cells, which, like Tregs, contribute to the microbial immune tolerance (Ghosh et al.,

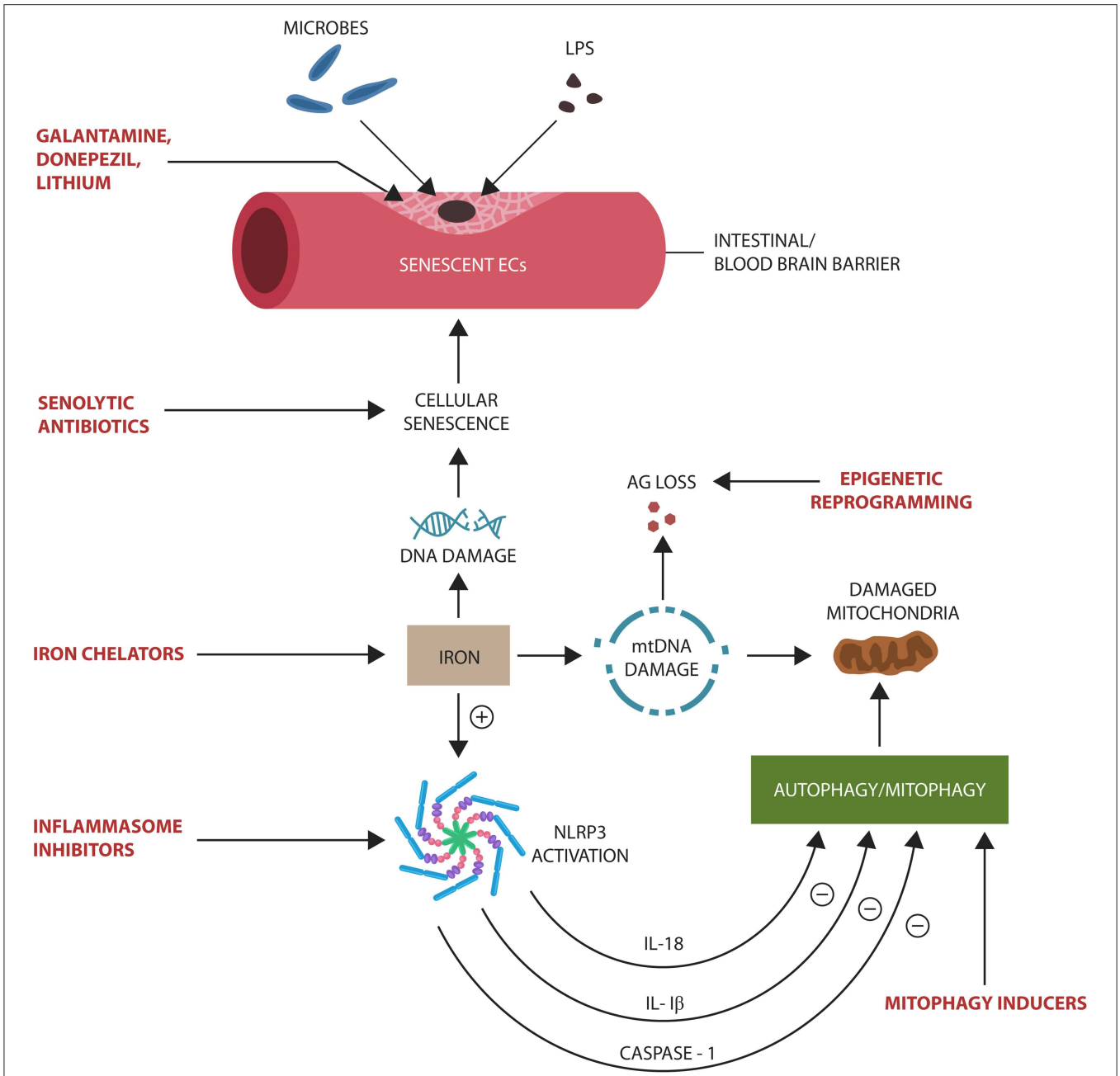


FIGURE 4 | Potential senotherapeutic interventions in AD and the steps at which they may operate. NLRP3 end products, IL-1β, IL-18, and caspase-1, inhibit autophagy and mitophagy, contributing to the accumulation of senescent cells and damaged mitochondria. These, in turn, alter biological barriers, enabling microbial translocation and AG loss. Excess iron induces DNA and mtDNA damage, activating NLRP3 with subsequent cellular senescence.

2013; van der Geest et al., 2016; Ip et al., 2017). Conversely, restoration of thymic function in older individuals or hormonal replacement may reverse immunosenescence, suggesting a novel therapeutic strategy. Indeed, preclinical studies reported that administration of EVs loaded with thymosin alpha 1 and melatonin restored the thymic function in older animals (Molinero et al., 2000; King and Tuthill, 2016; Wang et al., 2018).

Senescence and Iron

Age-related iron dysmetabolism, a phenomenon well-documented in AD, is closely connected to cellular senescence and the loss of AG (Kelleher and Soiza, 2013; Ward et al., 2014; Lane et al., 2015). Iron is known for inducing DNA damage and EC senescence that increases BBB permeability and the risk of microbial translocation (Won et al., 2011; Mollet et al., 2016) (**Figure 4**). Moreover, iron was demonstrated

to activate NLRP3 inflammasomes, linking this biometal to inflammation, dysfunctional mitochondria, and impaired mitophagy (Allen et al., 2013; Xiong et al., 2014; Nakamura et al., 2016). A component of iron–sulfur clusters (ISCs) and heme, iron has been demonstrated to alter mitochondrial glucose metabolism in response to pathogens (Horowitz and Greenamyre, 2010). For example, to deny microbes the access to glucose during infections, heme binds TLR-4, inducing hypoglycemia (Figueiredo et al., 2007; Weis et al., 2017). In AD however, hypoglycemia may be a double-edged sword as it may deepen the cellular energy crisis (Fong et al., 2016). This may explain the link between *P. gingivalis*, a heme-dependent pathogen, and T2DM, as well as the association of both with AD (Deshpande et al., 2010). In its attempt to extract heme, *P. gingivalis*, a facultative intracellular microbe, may damage not only cell membranes but also the mitochondrion, triggering a bioenergetic crisis and NLRP3-induced cellular senescence (Bullon et al., 2011). Moreover, age-related brain LPS elevation may trigger intracellular iron migration, an innate immune response to withhold iron from pathogens (Abreu et al., 2018; Ashraf et al., 2018). However, intracellular iron in proximity to redox biomolecules increases the risk of ROS generation, a known trigger of cellular senescence (Lopes et al., 2008; Streit and Xue, 2012). Conversely, the natural iron chelator lactoferrin binds LPS, deactivating NLRP3 (Drago-Serrano et al., 2012; Kruzel et al., 2017; Sfera et al., 2018). Interestingly, lactoferrin was recently identified as an AMP with anti-*P. gingivalis* properties, suggesting a therapeutic benefit in AD (Drago-Serrano et al., 2012; Kruzel et al., 2017).

The aging process, associated with intracellular iron retention, DNA damage, and impaired genomic repair, is a phenomenon we previously defined as ferrosenescence (Sfera et al., 2018). Along these lines, the levels of iron storage protein, ferritin, was found to be a reliable senescence marker, supporting the concept of ferrosenescence (Masaldan et al., 2018). This is in line with a novel hypothesis, suggesting that age-arelated increase in free iron pool resuscitates dormant microbes in the brain parenchyma (Pretorius et al., 2018). Furthermore, intracellular iron can promote Cdk5 nuclear export, tau hyperphosphorylation, and neuronal cell cycle activation (Engmann and Giese, 2009). On the other hand, iron chelation with deferoxamine was shown to have the opposite effect on tau, probably by facilitating Cdk5 nuclear reentry (Guo et al., 2013; Liu J. L. et al., 2018).

SENOTHERAPEUTICS: TARGETING SENESCENCE IN ALZHEIMER'S DISEASE

Senotherapeutics are pharmacological compounds, aiming at restoring senescent cells to non-senescent status or to trigger their apoptosis and clearance (Olivieri et al., 2018). These agents can be classified into senolytics that selectively eliminate senescent cells and senomorphics that delay or reverse senescence. Recent preclinical studies have shown that senotherapeutics can influence the course of age-related diseases, including AD (Kim and Kim, 2019). The agents described below include novel compounds and repurposed drugs with potential senotherapeutic properties.

Repurposed Galantamine, Donepezil, Lithium, and Fluoxetine

Galantamine and donepezil are cholinesterase inhibitors widely used in the treatment of AD. They function by inhibiting acetylcholine (ACh)-degrading enzymes and increasing the bioavailability of this neurotransmitter in brain cholinergic tracts. Both drugs were recently demonstrated to protect intestinal barrier and BBB, displaying potential senotherapeutic properties (Nakao et al., 2008; Zhang T. et al., 2015; Zhang Y. et al., 2017; Wazea et al., 2018). ACh-producing intestinal T cells have been reported to promote commensal microbes' immune tolerance, protecting against inflammation and barrier disruption (Dhawan et al., 2016). A recent study showed that alpha 7 nicotinic ACh receptor (α7nAChR) agonists function by deactivating NLRP3 in monocytes and microglia, promoting tolerance to commensals (Ke et al., 2017). Moreover, vagal nerve stimulation and transcranial direct current stimulation (tDCS) may present with senotherapeutic properties as they enhance cholinergic signaling (Chang et al., 2018).

Lithium, a drug used in the treatment of bipolar disorder, was reported to inhibit both mTOR and GSK3β, protecting the ECs of intestinal barrier and BBB (Motoi et al., 2014; Bosche et al., 2016; Steinbach et al., 2017; Martin et al., 2018). In addition, lithium modulates Cdk5, probably stabilizing neuronal cells in post-mitotic state (Jordà et al., 2005).

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) utilized in the treatment of major depressive disorder was demonstrated to inhibit NLRP3 and SASP, suggesting senotherapeutic properties (Diniz et al., 2016; Du et al., 2016). Indeed, a novel study reported that SSRIs, as a group, decrease the risk of conversion from mild cognitive impairment (MCI) to AD, likely by lowering cellular senescence (Bartels et al., 2018).

Mitophagy as a Senotherapeutic Strategy

Recent studies have associated defective mitochondria with cellular senescence as defects of these organelles activate NLRP3 (Liu Q. et al., 2018). On the other hand, elimination of defective mitochondria, mitophagy, delays senescence and lowers inflammation. Preclinical studies linked mitophagy enhancement to improved cognition, while accumulation of defective mitochondria was associated with AD pathology (Cai and Tammineni, 2016; Kerr et al., 2017).

Mitophagy as a therapeutic intervention was studied the most in PD in which defective mitochondria are cleared via phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and the E3 ubiquitin ligase parkin (PARK2) (Pickrell and Youle, 2015). Disruption of this autophagic pathway is a well-established pathogenetic mechanism in PD that may also play a role in AD (Martín-Maestro et al., 2016).

Another mitophagy system, associated with AD and traumatic brain injury (TBI), involves the inner mitochondrial membrane phospholipid, cardiolipin (Chu, 2018; Chao et al., 2019). Externalization of cardiolipin to the mitochondrial

surface was shown to activate neuronal mitophagy in rodents (Chu et al., 2013).

Mitophagy-inducing agents currently available include Mito-CP (3-carboxyl proxyl nitroxide), Mito-Metformin, and MitoTam (mitochondria-targeted tamoxifen) (Boyle et al., 2018; Hubackova et al., 2019). These compounds were demonstrated to activate mitophagy by various mechanisms, including depletion of ATP or adenine nucleotide translocase-2 (ANT2) (Singh et al., 2009; Zhang C. et al., 2015). Interestingly, several antibiotics, including quinolones, aminoglycosides, and β-lactams, were found to damage mitochondria, inducing cellular senescence (Kalghatgi et al., 2013; Stefano et al., 2017). Conversely, tetracycline derivatives, doxycycline, and minocycline were associated with the activation of mitophagy in ECs, suggesting protective effects for biological barriers (Dong et al., 2015; Xing et al., 2017).

Histone Deacetylase Inhibitors as Senotherapeutics

Histone deacetylases (HDACs) are enzymes involved in the epigenetic regulation of gene expression via histone proteins. HDACs have been involved in the pathogenesis of AD, and some HDAC inhibitors (HDACi) may present with cognition-enhancing properties (Xu et al., 2011). HDAC 1 and 2 inhibitors, including valproic acid (VPA), have been demonstrated to correct defective microglial phagocytosis, facilitating the elimination of molecular waste and dead cells (Datta et al., 2018). VPA, a drug utilized in the treatment of epilepsy and bipolar disorder, was recently shown to possess anti-HSV-1 actions, indicating a potential benefit in AD (Crespillo et al., 2016). In addition, this compound prevents LPS-induced ECs damage, protecting intestinal barrier and BBB (Chuang et al., 2014; Kasotakis et al., 2017).

Aside from VPA, other HDACis currently in clinical use include trichostatin A, sodium butyrate, and suberoylanilide hydroxamic acid (SAHA or vorinostat). SAHA is both an HDAC 6 inhibitor and an iron chelator, suggesting senotherapeutic properties (Hwang et al., 2015). SAHA is currently approved for the treatment of advanced primary cutaneous T cell lymphoma, but it also possesses anti-*P. gingivalis* properties, suggesting a therapeutic role in AD and periodontal disease (Yoshioka et al., 2003; Mann et al., 2007). Moreover, VPA and SAHA were recently found efficacious against *Mycobacterium tuberculosis*, an intracellular pathogen, suggesting efficacy against facultative intracellular microbes, including *P. gingivalis* (Rao et al., 2018).

It was recently reported that sirtuin 6 (SIRT6), a protein presenting with HDAC-like senotherapeutic properties, inhibits NF-κB and EC senescence, suggesting AD therapeutic benefits (Lappas, 2012; Zhao et al., 2016).

Iron Chelators in Cellular Senescence and Alzheimer's Disease

Iron is a pro-growth nutrient that accumulates in senescent cells, contributing to genomic instability and ROS generation (Killilea et al., 2003). A major component of the aging marker lipofuscin, iron is a driver of cellular senescence via mTOR activation and inhibition of mitophagy (Terman and Brunk, 1998; Höhn et al., 2010; Bayeva et al., 2012). Iron chelators, such as deferoxamine, are mTOR inhibitors demonstrated to lower the markers of senescence (Ohyashiki et al., 2009; Inoue et al., 2018). For example, intranasal administration of deferoxamine was found beneficial in animal models of AD, PD, and stroke (Fine et al., 2017). Moreover, as pathogens and host innate immune cells share the same iron pool, iron chelators deny this biometal to both, lowering microbial survival and ROS formation (Thompson et al., 2012). For this reason, iron chelator nanoparticles have been studied as AD therapeutics (Liu et al., 2010).

Another iron chelator with senotherapeutic properties, α-lipoic acid, is a BBB-crossing mitochondrial molecule with beneficial effects in AD (Baeeri et al., 2019; Camiolo et al., 2019). Preclinical studies linked this compound to mTOR inhibition and protection against brain ischemia (Gao et al., 2018). Other recent studies associated α-lipoic acid with intestinal barrier and BBB protection, indicating antitranslocation properties (Schreibelt et al., 2006; Varasteh et al., 2017).

The natural iron chelator lactoferrin, recently identified as an AMP, was found protective of ECs and biological barriers (Krylov et al., 2007; Wu et al., 2014). Inhibiting mTOR signaling and decreasing the iron pool, lactoferrin may be of potential therapeutic benefit in AD (Jenssen and Hancock, 2009; Zhang et al., 2014; van Splunter et al., 2018).

Inflammasome Inhibitors and Alzheimer's Disease

NLRP3 inhibitors are novel senotherapeutic agents that delay EC senescence and microbial translocation, suggesting beneficial effects in both AD and chronic inflammation (Yi, 2017; Yin et al., 2017; McAllister et al., 2018; Qi et al., 2018). Here, we focus primarily on NLRP3 inhibitors associated with the restoration of biological barriers.

MCC950, a diarylsulphonylurea inhibitor, lowers pyroptosis by selectively blocking NLRP3 inflammasomes, restoring the integrity of intestinal barrier (Fan et al., 2018; Perera et al., 2018). MCC950 also inhibits IL-1β, restoring BBB integrity (Lang et al., 2018). Interestingly, in PD, Cdk5 was shown to activate NLRP3, suggesting that inflammasome inhibitors may lower the detrimental effects of this kinase on neurons, preventing senescence and cell cycle engagement (Zhang et al., 2016). Indeed, to activate cytosolic NLRP3, Cdk5 must exit the nucleus, an event that triggers the neuronal cell cycle. Moreover, MCC950 has been shown to prevent immunosenescence of innate immune cells by blocking *P. gingivalis*-induced pyroptosis (Fleetwood et al., 2017).

INF 39, an acrylate NLRP3 inhibitor, was shown to decrease bowel inflammation in animal models by downregulating IL-1β, suggesting a therapeutic role against microbial translocation (Cocco et al., 2017; Pellegrini et al., 2018).

Milk fat globule membranes (MFGM) were reported to lower bacterial translocation in animal models by inhibiting NLRP3 and increasing the expression of intestinal

tight junctions, proteins opposing microbial translocation (Li Y. et al., 2018).

Short-chain fatty acids (SCFAs) were found trophic for IECs, restoring the integrity of intestinal barrier by functioning as energy sources and NLRP3 deactivators (Feng et al., 2018).

Statins were described as protective of ECs in both intestinal barrier and BBB via NLRP3 inhibition, reviving the debate about the benefit of these drugs in AD (Schreibelt et al., 2006; Krylov et al., 2007; Varasteh et al., 2017).

CONCLUSIONS

Commensal gut microbes live in symbiosis with the human host as long as they reside in the GI tract where they can be kept under control. Cellular senescence alters the integrity of biological barriers, allowing translocation and dissemination of gut microorganisms throughout the body tissues, including the brain. Operating "behind enemy lines," pathogens can gain control of host immune defenses and metabolism, triggering senescence and neurodegenerative pathology.

Senotherapeutics inhibit cellular senescence program, restoring the integrity of biological barriers. Moreover, the recent association of chronic *P. gingivalis* infection with both cellular senescence and AD emphasizes the importance of promptly treating periodontal disease.

Aging, a major risk factor of AD, is associated with senescent cell accumulation and SASP-induced pathology. In the CNS, senescent brain cells may display aberrant traits, including neuronal cell cycle activation and phagocytosis of viable neurons and synapses by aggressive glial cells. Since the molecular underpinnings of senescence, NF-kB-linked NLRP3 assembly, is modifiable, age-related neurodegenerative disorders could be epigenetically, pharmacologically, and immunometabolically influenced not only from within the CNS but also from the body periphery.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Gray Matter Densities in Limbic Areas and APOE4 Independently Predict Cognitive Decline in Normal Brain Aging

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Cross-sectional magnetic resonance imaging (MRI) studies reported significant associations between gray matter (GM) density changes in various limbic and neocortical areas and worst cognitive performances in elderly controls. Longitudinal studies in this field remain scarce and led to conflicting data. We report a clinico-radiological investigation of 380 cognitively preserved individuals who undergo neuropsychological assessment at baseline and after 18 months. All cases were assessed using a continuous cognitive score taking into account the global evolution of neuropsychological performances. The vast majority of Mini Mental State Examination (MMSE) 29 and 30 cases showed equal or worst performance at follow-up due to a ceiling effect. GM densities, white matter hyperintensities and arterial spin labeling (ASL) values were assessed in the hippocampus, amygdala, mesial temporal and parietal cortex at inclusion using 3 Tesla MRI Scans. Florbetapir positron emission tomography (PET) amyloid was available in a representative subsample of 64 cases. Regional amyloid uptake ratios (SUVr), mean cortical SUVr values (mcSUVr) and corresponding z-scores were calculated. Linear regression models were built to explore the association between the continuous cognitive score and imaging variables. The presence of an APOE-ε4 allele was negatively related to the continuous cognitive score. Among the areas studied, significant associations were found between GM densities in the hippocampus and amygdala but not mesial temporal and parietal areas and continuous cognitive score. Neither ASL values, Fazekas score nor mean and regional PET amyloid load was related to the cognitive score. In multivariate models, the presence of APOE-ε4 allele and GM densities in the hippocampus and amygdala were independently associated with worst cognitive evolution at follow-up. Our data support the idea that early GM damage in the hippocampus and amygdala occur long before the emergence of the very first signs of cognitive failure in brain aging.

Keywords: longitudinal study, cognition, magnetic resonance imaging, gray matter density, white matter hyperintensity, arterial spin labeling, hippocampus, amygdala

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INTRODUCTION

Cognitive trajectories in old age are variable ranging from cognitive stability to fluctuations over time and, in a limited number of cases, progressive worsening of neuropsychological performances corresponding to the pre-mild cognitive impairment (MCI) state. In a large sample from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort followed-up annually for 5 years, 40% of cases showed stable and high memory and executive function performances (successful agers) whereas 20% displayed progressive decline (declining agers; Lin et al., 2017). Similar data were obtained in Mexican Americans (Downer et al., 2017) and Korean aged over 65 years (Min, 2018). Although within normal age-adjusted performances, declining agers are thought to exhibit the first signs of cognitive frailty and are of particular interest for future therapeutic interventions. Most of the previous investigations in this field focused on the concept of preclinical AD searching to establish the predictive power of lesion burden, functional and structural brain changes in community-based samples of elderly controls. In this context, a combination of AD biomarkers in cerebrospinal fluid ($A\beta_{42}$, tau, and phospho-tau), non-invasive neuroimaging, and genetic risk factors have been investigated with promising but also conflicting data (for review, see Khan, 2018). When addressing the cognitive trajectories in old age, one should keep in mind the marked heterogeneity of elderly controls. Prior to 80 years, most of them did not correspond to the preclinical AD concept and their cognitive fate may be determined by a variety of other parameters such as significant vascular burden or limited cognitive reserve due to genetic or environmental factors (Li et al., 2017). But even within the theoretical framework of preclinical AD, there is substantial heterogeneity with respect to its neuropsychological definition (Epelbaum et al., 2017). Most of the longitudinal investigations in cognitively intact elderly individuals examined the evolution of neuropsychological parameters (working and episodic memory as well as executive abilities; Dubois et al., 2018; Rabin et al., 2018) or screening test (more frequently Mini Mental State Examination, MMSE) scores over time. However, cognitive performances may vary substantially in healthy elders with some of them declining and others remaining stable or even improving at follow-up. An accurate approach of cognitive evolution in this population needs to consider both improvement and decline of task performances in a wide range of cognitive tests over short time periods.

Among the different imaging techniques used to predict the cognitive evolution in elderly controls, amyloid positron emission tomography (PET) imaging and structural magnetic resonance imaging (MRI) changes are likely to address two distinct processes. Based on 1,209 cognitively intact individuals aged 50–95, Jack and collaborators showed that hippocampal volume loss may occur before abnormal amyloid PET occurrence. Unlike hippocampal volume decrease that starts at 30 years, and becomes significant after 60 years and is APOE4-independent, amyloid PET positivity occurred after 70 years and depended on the presence of APOEε4 alleles. These data indicated that Aβ accumulation arises in later life on a background of preexisting structural deficits that are associated with aging and not with amyloid pathology *per se* (Jack et al., 2015). Several observations pointed to the dissociation between neurodegeneration and amyloid pathology in normal aging and proposed two spatially distinct patterns of atrophy, a tau-related cortical thinning and Aβ-related hippocampal volume decrease, that may have a synergistic effect on subtle cognitive decline (Besson et al., 2015; Edmonds et al., 2015; Insel et al., 2015; Wang et al., 2015).

Given the recent progress in MRI analysis, several parameters are now available to explore the neuroanatomical substrates of the progressive transition from preserved cognition to the initial stages of cognitive deterioration. In normal aging, diffusion tensor imaging studies showed early fractional anisotropy decrease in the hippocampus and parahippocampal gyrus, supramarginal gyrus, frontotemporal lobes, mesial temporal lobes and anterior cigulate cortex (Hong et al., 2016; Lancaster et al., 2016). Data on gray matter (GM) are, however, more ambiguous. Regional GM decrements in right thalamus, left parahippocampal gyrus, inferior temporo-parietal lobules, anterior cingulum, and precentral gyrus have been documented (Lee et al., 2016; Fletcher et al., 2018; Squarzoni et al., 2018) but in certain cohorts GM densities were preserved or marginally affected in healthy controls (Hong et al., 2016; Takeuchi et al., 2017). The initial stages of cognitive deterioration may be related not only to structural but also functional changes that affect brain perfusion. In this line, we reported that reduced arterial spin labeling (ASL) in the posterior cingulate cortex at baseline is associated with the development of subtle neuropsychological deficits in healthy elderly controls (Xekardaki et al., 2015).

The present longitudinal study of a community-based cohort of highly educated elderly individuals explores the demographic, clinical and 3T MRI correlates of very subtle cognitive decline (prior to MCI) controlling for the presence of amyloid pathology. In order to obtain a global assessment of cognitive status without *a priori* hypotheses, we established a continuous cognitive score taking into account not only AD-related cognitive functions and considering both improvement and decline of neuropsychological performances at follow-up. Using univariate and multivariate linear regression models controlled for demographic variables (age, gender, education), MMSE scores at baseline, amyloid load, and Fazekas score of white matter lesion severity, we explored the association between subtle cognitive changes and patterns of GM volumes and ASL values in limbic and temporo-parietal areas (Bharath et al., 2017; Zanchi et al., 2017).

MATERIALS AND METHODS

Population

The protocol was approved by the Ethics Committee of the Geneva University Hospitals of Geneva. All experimental procedures were carried out in accordance with the approved guidelines and with the principles of the Declaration of Helsinki. All participants were given written informed consent prior to inclusion. These community-based cases were recruited *via* advertisements in local newspapers and media. All participants had normal or corrected-to-normal visual acuity. Past hearing

problems were identified as a part of the medical interview (including both subjects and their proxies). All cases with such problems were *a priori* excluded. Audition was tested by standard audiologic tests including self-report and speech in noise perception in all cases during clinical routine medical examination. Cases with self-report of hearing loss and altered speech in noise perception were addressed in specialized consultation and were not considered for further investigations. The education level was defined according to the Swiss scholar system, where level 1 = less than 9 years (primary school), level 2 = between 9 and 12 years (high school) and level 3 = more than 12 years (university). To control for the confounding effect of cardiovascular diseases, individuals with subtle cardiovascular symptoms and a history of stroke and transient ischemic episodes were also excluded from the present study. The inclusion period for control subjects was from October 2014 to March 2016.

The final sample included 380 elderly controls: 232 (61.1%) women and 148 (38.9%) men, aged 74.2 ± 4.1 (mean ± SD) ranging from 68.6 to 90.0 years, all assessed with structural and resting state fMRI at baseline (Zanchi et al., 2017).

Neuropsychological Assessment

Participants were evaluated at inclusion with an extensive neuropsychological battery, including the MMSE (Folstein et al., 1975), the Hospital Anxiety and Depression Scale (HAD; Zigmond and Snaith, 1983), and the Lawton Instrumental Activities of Daily Living (IADL; Barberger-Gateau et al., 1992). Cognitive assessment included: (a) attention [Digit-Symbol-Coding (Wechsler, 1997a), Trail Making Test A (Reitan, 1958)]; (b) working memory [verbal: Digit Span Forward (Wechsler, 1997b), visuospatial: Visual Memory Span (Corsi; Milner, 1971)]; (c) episodic memory [verbal: RI-48 Cued Recall Test (Buschke et al., 1997), visual: Shapes Test (Baddley et al., 1994)]; (d) executive functions [Trail Making Test B (Reitan, 1958), Wisconsin Card Sorting Test (WCST; Heaton et al., 1993) and Phonemic Verbal Fluency Test (Cardebat et al., 1990)]; (e) language (Boston Naming Test; Kaplan et al., 1983); (f) visual gnosis (Ghent, 1956); and (g) praxis ideomotor (Schnider et al., 1997), reflexive (Poeck, 1985), and constructional (Consortium to Establish a Registry for Alzheimer's Disease, CERAD), figures copy (Welsh et al., 1994).

In agreement with the criteria of Petersen et al. (2001), participants with a CDR score of 0.5 but no dementia and a score exceeding 1.5 standard deviations (SDs) below the age-appropriate mean in any of the previously mentioned tests were classified as MCI and were excluded. Participants who met DSM-IV diagnostic criteria of dementia on the basis of the neuropsychological and clinical assessments were also excluded. Participants with neither dementia nor MCI were classified as cognitively healthy older controls and underwent full neuropsychological assessment once at follow-up, on average 18 months later, with the same neuropsychological battery.

APOE Assessment

Whole blood samples were collected at baseline for all subjects for APOE genotyping. Standard DNA extraction was performed using either 9 ml EDTA tubes (Sarstedt, Germany) or Oragene Saliva DNA Kit (DNA Genotek, Inc., Ottawa, ON, Canada) which were stored at −20°C. APOE genotyping was done on the LightCycler (Roche Diagnostics, Basel, Switzerland) as described previously (Nauck et al., 2000). Subjects were classified according to the presence of an APOEε4 allele (ε4/ε3, ε3/ε3, ε3/ε2 carrier).

MRI Imaging

Imaging data were acquired on a 3T MRI Scanner (TRIO Siemens Medical Systems, Erlangen, Germany). A high-resolution T1-weighted anatomical scan (magnetization prepared rapid gradient echo (MPRAGE), 256 × 256 matrix, 176 slices 1 mm isotropic, TR = 2,300 ms, TE = 2.27 ms,) was collected as well as a pulsed ASL sequence [64 × 64 matrix, 24 slices, voxel size 3.44 × 3.44 × 5 mm^3, TE 12 ms, TR 4,000 ms, inversion time (TI) 1,600 ms]. Additional sequences included axial fast spin-echo T2-weighted imaging (4,000/105, 30 sections, 4-mm section thickness), susceptibility weighted imaging (28/20, 208 × 256 × 128 matrix, 1 × 1 × 1 mm^3 voxel size) were performed to exclude brain disease, such as ischemic stroke, subdural hematomas, or space-occupying lesions.

Assessment of Gray Matter Volumes

3DT1 MRIs were preprocessed with the FSL software package[1], according to the standard procedure. The essential processing steps included brain extraction with the FSL Brain Extraction Tool[2], tissue-type segmentation with the FMRIB Automated Segmentation Tool[3], nonlinear transformation into Montreal Neurological Institute reference space, and creation of a study-specific GM template to which the native GM images were then nonlinearly reregistered. The modulated segmented images were then smoothed with an isotropic Gaussian kernel with a width of 2 mm. Furthermore, we created a mask for the bilateral mesial temporal cortex, hippocampus, amygdala, caudate nuclei, and parietal lobes that was then applied to the GM image of the study-specific template, and we obtained GM density values in each area of interest.

Arterial Spin Labeling

The reconstructed relCBF ASL perfusion images were spatially normalized using a linear spatial alignment from ASL raw data to the individual high-resolution 3DT1 image, followed by the application of the non-linear spatial registration determined in the pre-processing of the 3DT1 data. These spatial transformations were then applied to the relCBF maps calculated directly on the MRI scanner. This two-steps approach results in a non-linear spatial registration of the ASL relCBF map into the MNI space. We then calculated the relCBF values in each area of interest.

In addition to the GM and ASL analysis, white matter lesion severity was analyzed on T2-weighted images according to the established Fazekas scale (Fazekas et al., 1987).

Amyloid PET Imaging With (18)F-Forbetapir

Florbetapir images were acquired 50–60 min after injection for 77 subjects. Seventy PET data were acquired on a Discovery

[1] http://www.fmrib.ox.ac.uk/fsl/
[2] http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BET
[3] http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/fast

PET/CT 710 scanner (GE Healthcare) and 7 PET imaging were performed using a Biograph^{TM} mCT scanner (Siemens). All studies were quantitatively evaluated using the Amyvid BRASS commercial automated functional brain analysis software (Hermes Medical Solutions AB, Stockholm, Sweden). Regional amyloid uptake ratios (SUVr), mean cortical SUVr values (mcSUVr) and z-score (number of SDs from the healthy control SUVr in the BRASS template) were calculated relative to the cerebellum.

Statistics

Demographic and neuropsychological data were compared between the two visits with paired *t*-test and Wilcoxon matched-pairs signed rank test. The significance level was set at $P < 0.05$ but was corrected to $P < 0.0079$ for multiple testing by using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

The subtle cognitive decline continuous score was defined as follows. Most of the cognitive performances, discrete or continuous, cannot be linearly combined by adding the individual scores to a unique composite cognitive score. Thus all values were converted to z scores. Subsequently, we summed the number of cognitive tests at follow-up with performances at least 0.5 SD higher compared with the first evaluation, leading to the number of tests with improved performances (range, 0–14). Similarly, we summed the number of cognitive tests at follow-up with performances at least 0.5 SDs lower compared with the first evaluation, yielding the number of tests with decreased performances (range, 0–14). Finally, the number of tests with improved minus the number of tests with decreased performances results in a final continuous cognitive score.

Simple and multiple linear regression models were used to identify predictors of the continuous cognitive score (dependent variable) including GM volumes, ASL values and potential confounders such as age, gender, education levels, MMSE at baseline, Fazekas score, and APOE genotyping. Full models and models with only the significant variables were obtained by stepwise backward selection process. Each model was run for all four regions independently (hippocampus, middle temporal gyrus, parietal cortex, amygdala). To examine whether working memory or episodic memory changes were associated with our independent variables, we run the same regression models using the neuropsychological tests (z-score) used for these functions (Digit Span Forward, Visual Memory Span, RI-48 Cued Recall Test and Shapes Test) as the dependent variable. The same models were also run while adding normalized cortical amyloid volume assessed by florbetapir PET expressed either as a continuous variable or a binary score (below vs. above or equal to SUVr 1.2).

We also added to the above simple and multiple linear regression models an Apoe4*GM volume interaction term and subsequently built an ANCOVA model to perform a power analysis using the PASS version 13 software (PASS 13. NCSS, LLC. Kaysville, Utah, USA[4], 2014).

[4]www.ncss.com

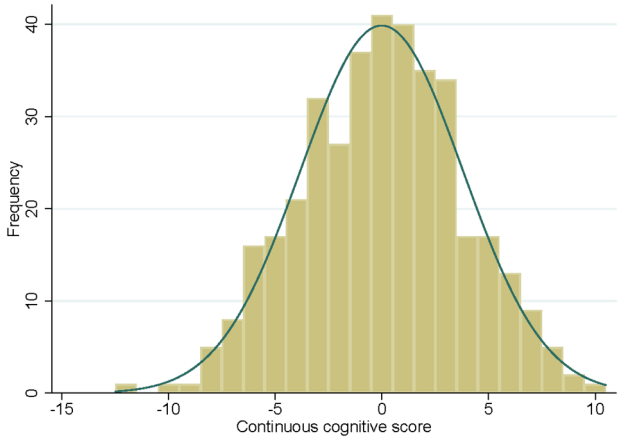

FIGURE 1 | Histogram of the continuous cognitive score at follow-up with the corresponding normal density curve.

All other statistics were performed with the STATA statistical software, Version 15.1 (StataCorp, College Station, TX, USA, 2017).

RESULTS

Sample Description

The distribution of the continuous cognitive score at follow-up is illustrated in **Figure 1**. Education levels were distributed as follows: 63 (16.6%) level 1 (primary school), 175 (46.0%) level 2 (high school) and 142 (37.4%) level 3 (university). Sixty-five (17.1%) participants had at least one allele APOEε4. Mean MMSE score was of 28.5 ± 1.3 (mean ± SD) ranging from 24.0 to 30.0.

Cognition

Univariate analysis showed that MMSE at baseline was negatively related to the continuous cognitive score [regression coefficient: −0.48 (−0.78, −0.17)] meaning that the highest is the MMSE score the lower is the cognitive score (implying that the most preserved cases are at higher risk to deteriorate; see **Figure 2A**). This analysis also showed that the presence of an APOEε4 allele was associated with significantly lower continuous cognitive scores [regression coefficient: −1.09 (−2.13, −0.04); see also **Figure 2B**]. Neither age nor gender or education was related to the cognitive outcome in this cohort.

Cognition and GM Densities

Among the areas studied, significant positive associations were found between GM densities in the hippocampus and continuous cognitive score [regression coefficient: 12.54 (1.99, 23.10); see **Figure 3** left panel]. This was also the case for the amygdala [regression coefficient: 11.04 (2.24, 19.84); see **Figure 3** right panel] but not for mesial temporal and parietal gyrus. Importantly, ASL values in the areas studied were not related to the cognitive score. This was also the case for the Fazekas score in all of the areas studied.

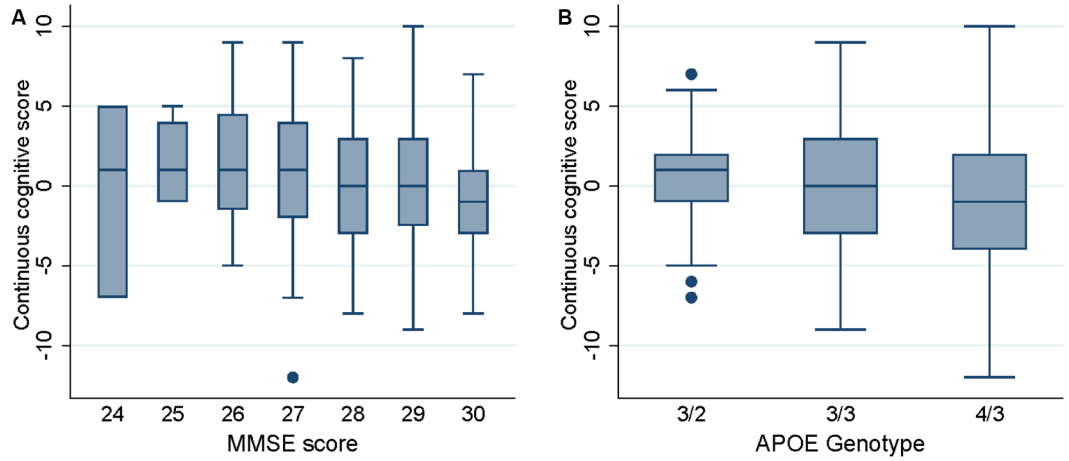

FIGURE 2 | Box plots of the continuous cognitive score at follow-up as a function of Mini Mental State Examination (MMSE) score (panel **A**) and APOE genotype (panel **B**).

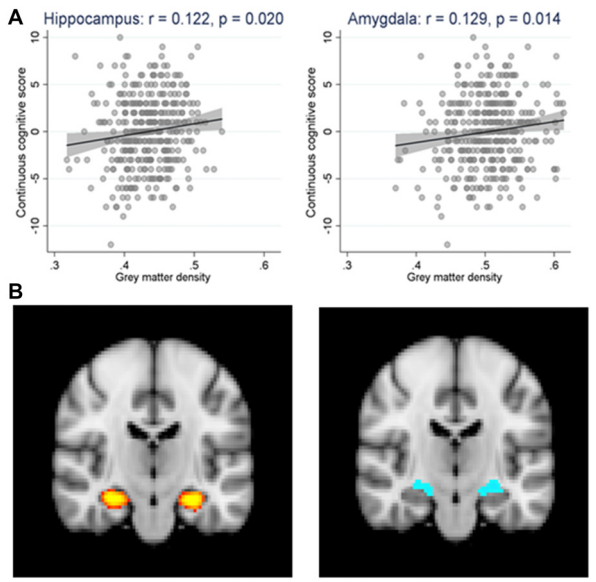

FIGURE 3 | (A) Scatter plot and linear regression (along with its 95% confidence bands) of the continuous cognitive score (cgs) over the gray matter density (gmd) in the hippocampus (cgs = −5.44 + 12.55* gmd, $p = 0.020$) and the amygdala (cgs = −5.57 + 11.04* gmd, $p = 0.014$) regions. $N = 362$. r = Pearson's correlation coefficient. **(B)** The regions of interest are highlighted in yellow for the hippocampus (left panel) and blue for the amygdala (right panel) in brain magnetic resonance imaging (MRI) scan.

In multivariate models taking into account MMSE scores, APOE genotyping, and the three MRI variables (GM densities, ASL values, Fazekas score), the presence of APOEε4 allele, higher MMSE score at baseline and GM densities in the hippocampus and amygdala were all associated with worst cognitive evolution at follow-up (**Table 1**). Although significant, the model including these parameters explained 7% of the cognitive variability. To explore a possible ceiling effect of MMSE score in our sample, we examined the association between MMSE scores at baseline and its evolution at follow up (increment or decrement; **Table 2**). The vast majority of MMSE 29 and 30 cases showed equal or worst performance at follow-up due to a ceiling effect. When working or episodic memory tests alone were used as dependent variable, no significant association was found with MRI variables (data not shown).

The Apoe4*GM volume interaction term was not statistically significant (ANCOVA: hippocampus: $p = 0.597$; amygdala: $p = 0.623$). A *post hoc* power analysis yield equivalent results for both areas. A total sample of 798 would be needed to achieve 84% power to detect differences among the means vs. the alternative of equal means using an F-test with a 0.050 significance level.

Sub-sample With Amyloid PET

Sixty-four participants underwent an amyloid PET. They did not differ in respect to age, gender, education, MMSE score and continuous cognitive score compared to the 298 cases without amyloid PET (see **Table 3**).

Normalized cortical amyloid volume in the hippocampus and the amygdala expressed either as a continuous variable (hippocampus: $p = 0.714$; amygdala: $p = 0.566$) or a binary score ($p = 0.340$; $p = 0.304$) was not associated with cognitive evolution.

DISCUSSION

Our data in a community-based cohort of highly educated controls indicate reveal that GM densities in the hippocampus and amygdala but not mesial temporal and parietal gyrus are associated with worst global cognitive performances in healthy controls. As recently reported, amyloid load was not related to cognitive changes at follow-up in the present series. This was also the case for white matter hyperintensities (Fazekas score) and ASL values, two MRI variables frequently cited as correlates of cognitive impairment in

TABLE 1 | Multiple linear regression models predicting the continuous cognitive score (dependent variable) with only the significant variables obtained by stepwise backward selection process and adjusted for the main confounders [Mini Mental State Examination (MMSE) at baseline, APOE genotyping, ASL, Gray matter index and FAZEKAS score].

	Reduced model				
Region	**Coef**	**95% CI**			**P**
HIPPOCAMPUS					
APOE Genotype					
3/2	0.219	−1.021	–	1.460	0.728
3/3	0.000				–
4/3	−1.051	−2.092	–	−0.011	0.048
MMSE	−0.499	−0.806	–	−0.191	0.002
ASL	0.002	−0.001	–	0.004	0.135
Gray matter index	12.701	2.090	–	23.311	0.019
FAZEKAS					
Absent	0.000				–
Mild	0.419	−0.464	–	1.302	0.352
Moderate	0.804	−0.375	–	1.983	0.181
Severe	−0.327	−1.980	–	1.325	0.697
AMYGDALA					
APOE Genotype					
3/2	0.133	−1.112	–	1.377	0.834
3/3	0.000				–
4/3	−1.044	−2.079	–	−0.008	0.048
MMSE	−0.493	−0.800	–	−0.186	0.002
ASL	0.002	−0.0002	–	0.003	0.078
Gray matter index	10.575	1.699	–	19.452	0.020
FAZEKAS					
Absent	0.000				–
Mild	0.432	−0.448	–	1.312	0.335
Moderate	0.748	−0.426	–	1.923	0.211
Severe	−0.292	−1.942	–	1.359	0.728

A different model was run for each of the two regions.

brain aging (Son et al., 2012; Xekardaki et al., 2015; Arvanitakis et al., 2016; De Vis et al., 2018). In conjunction with previous studies in normal aging, these findings suggest that changes in GM densities in limbic areas is the most reliable correlate of cognitive decline at the very early stages of brain aging.

GM loss in limbic areas has been often associated with time to progression from MCI to AD (Atiya et al., 2003; Kantarci and Jack, 2003; Younes et al., 2014). Early studies have already reported that the decrease of GM density in the hippocampus and amygdala may predict subsequent cognitive decline at the pre-MCI state (Jack et al., 2010; Squarzoni et al., 2012; Edmonds et al., 2015). In the same line, baseline measures of the hippocampus and amygdala in preclinical AD patients predict subsequent development of MCI (Grundman et al., 2002; den Heijer et al., 2006; Griffith et al., 2013; Guderian et al., 2015). Younes and collaborators also reported that hippocampal and amygdala GM atrophy occurs 2–4 years prior to the first signs of AD (Younes et al., 2014). However, the decrease of GM density in these areas varies substantially in preclinical AD cases reflecting the heterogeneity of the structural damage at this stage of the degenerative process (Lauriola et al., 2017; Perrotin et al., 2017). Two recent longitudinal studies in large community-based cohorts led to conflicting data. In the Rotterdam study of 3,264 cases, hippocampal volume was not associated with cognitive decrement at 5 years (Vibha et al., 2018). In contrast, Fletcher et al. (2018) found that baseline hippocampal volumes had significant incremental effects on cognitive decline in 460 cognitively preserved elders. The present data indicate that GM densities in the hippocampus and amygdala are significant predictors of the continuous cognitive score.

Cognitive variations in healthy elders within the normal range may be determined by demographic factors, basal cognition, and APOE genotyping that were not systematically taken into account in multivariate models. In fact, male gender, low level of education, and mainly the presence of APOE4 allele have been associated with worst cognitive trajectories in non-demented elders (Bretsky et al., 2003; Honea R. et al., 2009; Honea R. A. et al., 2009; Zehnder et al., 2009; Haller et al., 2017; Li et al., 2017; Lin et al., 2017) but negative or ambiguous data have also been reported (Van Gerven et al., 2012; López et al., 2017; Min, 2018). In the present study, higher MMSE scores are associated with higher risk of cognitive decline in this series. At first glance, this finding may be surprising. Early contributions showed that single MMSE measures do not allow for identifying MCI subjects who convert to AD (Arevalo-Rodriguez et al., 2015). Moreover, higher MMSE scores at AD diagnosis is associated with faster cognitive decline due to the rapid accumulation of neuropathological changes after diagnosis (Contador et al., 2017). Most of our cases scored 28 and above at baseline. As expected, the vast majority of MMSE 29 and 30 cases showed equal or worst performance at follow-up due to a ceiling effect. Interestingly, the rare cases with MMSE scores of 25 and 26 remained stable or

TABLE 2 | Association between MMSE scores at baseline and its evolution at follow up (increment or decrement).

	Delta MMSE score (follow-up minus baseline)											
	Worsening						Improvement					
Baseline MMSE	**−5**	**−4**	**−3**	**−2**	**−1**	**0**	**1**	**2**	**3**	**4**	**5**	**Total**
24	0	0	0	0	0	1	0	1	1	0	0	3
25	0	0	0	0	0	1	2	2	2	0	0	7
26	0	0	0	0	1	3	3	6	2	1		16
27	0	0	0	2	7	9	15	3	3			39
28	0	0	0	8	16	28	31	7				90
29	1	3	4	17	25	43	27					120
30	0	4	7	12	36	28						87
Total	1	7	11	39	85	113	78	19	8	1	0	362

The gray zone of the table corresponds to non-existing values.

TABLE 3 | Comparison of baseline characteristics of cases with and without amyloid positron emission tomography (PET).

	PET		Total	*P*
	No *N* = 298	Yes *N* = 64	*N* = 362	
Age at evaluation	74.2 ± 4.1	74.1 ± 4.0	74.2 ± 4.1	0.833
Gender				0.625
F	179 (60.1%)	39 (60.9%)	218 (60.2%)	
M	119 (39.9%)	25 (39.1%)	144 (39.8%)	
NSC				0.557
<9	50 (16.8%)	11 (17.2%)	61 (16.9%)	
9–12	136 (45.6%)	28 (43.8%)	164 (45.3%)	
>12	112 (37.6%)	25 (39.1%)	137 (37.8%)	
Génotype APOE				0.402
3/2	36 (12.1%)	5 (7.8%)	41 (11.3%)	
3/3	212 (71.1%)	47 (73.4%)	259 (71.5%)	
4/3	50 (16.8%)	12 (18.8%)	62 (17.1%)	
MMSE	28.5 ± 1.3	28.5 ± 1.2	28.5 ± 1.3	0.600
Continuous cognitive score	−0.0 ± 3.9	0.2 ± 3.5	0.0 ± 3.8	0.701

even improved at follow-up pointing to the selection of highly educated cognitively preserved volunteers in this study. From this point of view, our cases cannot be compared to those with NIA-AA preclinical stage 3 that usually display worst cognitive outcomes over time. In respect to APOE genotyping, several studies suggested that the presence of an APOEε4 allele is related to longitudinal changes in medial temporal cortical thickness, and hippocampal atrophy rates (Donix et al., 2010; Lu et al., 2011; Reiter et al., 2017). The independent contribution of APOE genotyping, hippocampal and amygdala atrophy on worst cognitive performances in healthy controls has been suggested by Honea R. et al. (2009) and Squarzoni et al. (2012) in their cross-sectional investigations. However, the association between APOEε4 allele and cognition at the pre-MCI stages is still a matter of debate with some studies showing no association between APOEε4 and memory performances or hippocampal volume in cognitively normal individuals (Protas et al., 2013; Jack et al., 2015; Lupton et al., 2016). When considering the continuous cognitive score that provides an overall assessment of high cortical functions, our data reveal that APOEε4 genotype and GM density in the hippocampus and amygdala are all independent predictors of cognitive decrement in healthy elderly people. Importantly, no such association was found when isolated tests of working or episodic memory were taken into account indicating that detailed neuropsychological exploration of all cognitive abilities in cognitively preserved individuals at baseline where the known patterns of cognitive vulnerability established in MCI could be not applicable.

Our negative data should be also discussed. Adding to recent lines of evidence, PET amyloid load (measured both in neocortical areas as well as hippocampus and amygdala) did not predict subtle cognitive changes in the present cohort (Dubois et al., 2018). Current evidence about the role of amyloid accumulation in cognitively preserved controls remain ambiguous. Although early data suggested that elevated amyloid levels at baseline (SUVr > 1.5) were associated with greater cognitive decline at follow-up (Petersen et al., 2016), more recent contributions indicated that PIB PET β-amyloid's relationship to cognitive decline was nonlinear being more prominent at lower β-amyloid levels (Knopman et al., 2018). The INSIGHT-pre AD data published recently showed no association between this parameter and cognitive fate at 30-month follow-up in healthy controls (Dubois et al., 2018). Our observations agree with this latter viewpoint implying that the detrimental effect of amyloid accumulation in cognitively preserved elders is at the best marginal. However, this observation should be interpreted with caution since our cases showed low amyloid accumulations with SUVr values >1.2 only in 16/64 of cases. Only two cases exceeded the cut-off value of 1.5. It is thus possible that amyloid burden was too low to produce significant cognitive impact in this cohort. The association of GM densities with cognitive performances is limited to the hippocampus and amygdala and did not involve mesial temporo-parietal association areas. In a previous study, Lancaster et al. (2016) reported that DTI variables in mesial temporal lobe is associated with a decline of episodic memory at 3-year follow-up in healthy controls. In elderly persons with subjective cognitive impairment, Hong et al. (2016) reported decreased fractional anisotropy in supramarginal gyrus and frontotemporal lobes in the absence of GM atrophy. Second, the low to moderate white matter hyperintensities burden (as measured by the Fazekas score) did not impact on the cognitive fate of the present cases. This finding is consistent with a recent study by Moon and collaborators (Moon et al., 2017) who showed that baseline white matter hyperintensities did not predict cognitive decline at follow-up to 3 years in non-demented older adults with memory complaints. Importantly and unlike our cases, the positive association between white matter hyperintensities and neuropsychological decline reported in earlier studies mainly concerned individuals with high Fazekas scores (Son et al., 2012; Arvanitakis et al., 2016). In the same line, ASL values did not predict continuous cognitive score in any of the areas studied. Contrasting with this observation, two recent studies showed that ASL decrease in medial frontal, anterior and posterior cingulate cortex predicts cognitive function in healthy elderly controls (Xekardaki et al., 2015; De Vis et al., 2018). Taken together, these observations imply two distinct MRI correlates of subtle cognitive deficits prior to MCI status: GM loss in the hippocampus and amygdala but also white matter microstructure and brain perfusion changes in neocortical areas outside the mesial temporal and parietal lobes. Such hierarchical pattern of MRI changes in brain aging is consistent with the idea that early GM damage in the hippocampus and amygdala is evident long before the emergence of the very first signs of cognitive failure in brain aging whereas more subtle white matter and functional changes, usually not detected in routine clinical settings, are present in neocortical association areas at the same time period (Younes et al., 2014; Zanchi et al., 2017).

Strengths of the present study include its longitudinal design in a community-based setting, detailed neuropsychological testing at inclusion and follow-up, use of continuous cognitive score that takes into account both improvement and worsening

of cognitive performances in each neuropsychological test, consideration of major confounders such as amyloid burden, APOE genotyping and MMSE score at baseline, and inclusion of ASL measures of cerebral perfusion. However, some limitations should also be considered. In the absence of longer follow-up, the decrease of the continuous cognitive score does not represent a marker of incipient dementia. No CSF measures of tau and Aβ protein were available in this work so that the real extent of AD pathology remains unknown. Our multivariate model explains only 7% of the cognitive variability. When interpreting this modest percentage, one should keep in mind that, in contrast to MCI and AD cases, healthy controls display an impressive variability in MRI parameters (Lauriola et al., 2017; Perrotin et al., 2017). The combination of multiple MRI modalities including ASL and DTI data in other neocortical areas but also CSF or PET assessment of tau pathology is warranted to improve the performance of imaging-based models of cognitive prediction in normal brain aging.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The protocol was approved by the Ethics Committee of the Geneva University Hospitals of Geneva. All experimental procedures were carried out in accordance with the approved guidelines and with the principles of the Declaration of Helsinki. All participants were given written informed consent prior to inclusion.

AUTHOR CONTRIBUTIONS

FH, VG, SH and PG: conceived the study. CR, M-LM and SH: recruited. CR, M-LM and PG: neuropsychology supervising. VG, M-LM and SH: imaging. CR, M-LM and FH: data preparation. FH, CR, SH, VG, M-LM and PG: analyzed the data and manuscript writing.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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The Precursor to Glutathione (GSH), γ-Glutamylcysteine (GGC), Can Ameliorate Oxidative Damage and Neuroinflammation Induced by Aβ$_{40}$ Oligomers in Human Astrocytes

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Glutathione (GSH) is one of the most abundant thiol antioxidants in cells. Many chronic and age-related diseases are associated with a decline in cellular GSH levels or impairment in the catalytic activity of the GSH biosynthetic enzyme glutamate cysteine ligase (GCL). γ-glutamylcysteine (GGC), a precursor to glutathione (GSH), can replenish depleted GSH levels under oxidative stress conditions, by circumventing the regulation of GSH biosynthesis and providing the limiting substrate. Soluble amyloid-β (Aβ) oligomers have been shown to induce oxidative stress, synaptic dysfunction and memory deficits which have been reported in Alzheimer's disease (AD). Calcium ions, which are increased with age and in AD, have been previously reported to enhance the formation of Aβ$_{40}$ oligomers, which have been casually associated with the pathogenesis of the underlying neurodegenerative condition. In this study, we examined the potential beneficial effects of GGC against exogenous Aβ$_{40}$ oligomers on biomarkers of apoptosis and cell death, oxidative stress, and neuroinflammation, in human astrocytes. Treatment with Aβ$_{40}$ oligomers significantly reduced the cell viability and apoptosis of astrocyte brain cultures and increased oxidative modifications of DNA, lipids, and protein, enhanced pro-inflammatory cytokine release and increased the activity of the proteolytic matrix metalloproteinase enzyme, matric metalloproteinase (MMP)-2 and reduced the activity of MMP-9 after 24 h. Co-treatment of Aβ$_{40}$ oligomers with GGC at 200 μM increased the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) and led to significant increases in the levels of the total antioxidant capacity (TAC) and GSH and reduced the GSSG/GSH ratio. GGC also upregulated the level of the anti-inflammatory cytokine IL-10 and reduced the levels of the pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and attenuated the

changes in metalloproteinase activity in oligomeric $A\beta_{40}$-treated astrocytes. Our data provides renewed insight on the beneficial effects of increased GSH levels by GGC in human astrocytes, and identifies yet another potential therapeutic strategy to attenuate the cytotoxic effects of Aβ oligomers in AD.

Keywords: Alzheimer's disease, glutathione, oxidative stress, antioxidants, dementia

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia affecting the elderly. Extracellular deposition of β-amyloid (Aβ plaques), intraneuronal tau accumulation, inflammation (activated astrocytes and microglia), and neuronal loss are all consistent pathological features of the disease (Porquet et al., 2015). Unlike Aβ plaques, inflammation correlates with neuronal loss and cognitive decline in AD, suggesting it plays an important role in disease progression (Wang et al., 2015; Wilkins et al., 2015).

It is well established that Aβ peptides existed in both fibrillary and non-fibrillar forms (Poduslo and Howell, 2015; Zhang et al., 2015). Soluble oligomeric Aβ aggregates have been shown to bind specifically to synapses in differentiated hippocampal neuronal cultures. These oligomers are capable of disrupting long-term potentiation, a classic experimental paradigm for memory and synaptic plasticity (Izzo et al., 2014; Nguyen and Derreumaux, 2014). Small soluble Aβ oligomers are now considered the primary neurotoxic entity in AD. Several studies have shown that both oligomeric $A\beta_{42}$ and $A\beta_{40}$ are both neurotoxic using both human and murine neuronal cell cultures. Moreover, there is a strong association between the level of soluble oligomeric Aβ, and the severity of synaptic loss and cognitive dysfunction in AD when compared to their fibrillar counterpart (Lesne, 2014; Nguyen et al., 2014; Wang et al., 2015). Similarly, a number of early-onset familial AD mutations display a more aggressive disease course and a greater propensity for Aβ to form soluble oligomeric Aβ aggregates (Ferreira and Klein, 2011; Streltsov et al., 2011; Wilcox et al., 2011). While several studies have examined the neurotoxic potential of $A\beta_{42}$ in several models, little is known about the effects of $A\beta_{40}$ oligomers in human astrocytes.

Glutathione (GSH) is an important endogenous antioxidant found in millimolar concentrations in the brain. GSH levels have been shown to decrease with ageing and in several age-related degenerative diseases and AD in particular (Harris et al., 2015; Ilyas and Rehman, 2015; Romero-Haro and Alonso-Alvarez, 2015). Soluble oligomeric Aβ has been shown to induce oxidative stress and has been proposed to play a central role in the oxidative damage detected in AD brain (Xu et al., 2014). It has been shown that administration of γ-glutamylcysteine (GGC) increases cellular levels of GSH, circumventing the regulation of GSH biosynthesis by providing the limiting substrate (Nakamura et al., 2012; Quintana-Cabrera et al., 2012).

Whilst extracellular Aβ plaques, NFT, inflammation in the form or reactive astrocytes and microglia, and neuronal loss are all consistent pathological features of AD, a mechanistic link between these factors is yet to be clarified. Although most of the past research has focused on fibrillar Aβ, soluble oligomeric Aβ species are now considered to be of major pathological importance in AD. GGC is a dipeptide which exhibits potent antioxidant properties in several experimental models (Lai et al., 2008; Quintana-Cabrera et al., 2012). It serves as an essential cofactor for the antioxidant enzyme glutathione peroxidase (GPx) and is a precursor for GSH synthesis (Quintana-Cabrera et al., 2012). The metal-chelating properties of GGC have also been demonstrated previously (Salama et al., 2016).

In this study, we evaluated the protective role of up-regulation of GSH by GGC against biomarkers of apoptosis and cell death, oxidative stress, and neuroinflammation against oligomeric $A\beta_{40}$ in primary human astrocyte cell cultures.

MATERIALS AND METHODS

Cell Cultures

Human adult brain tissues were collected following surgical resection with both informed and written consent from patients who underwent surgery at the Centre for Mininally Invasive Neurosurgery, Prince of Wales Private Hospital, NSW, Australia. Healthy brain specimens were also acquired from patients during surgery for their tumor. A portion of the surgically resected brain tumor and healthy tissue sample was snap-frozen in liquid nitrogen immediately and stored at −80°C for prolonged storage for future use. Astrocytes were prepared from the mixed brain cell cultures using a protocol previously described by Guillemin et al. (2001).

Human astrocytes were pre-incubated for 15 min with 200 μM GGC. Afterward, cells were treated with oligomeric $A\beta_{40}$ (10 nM, see **Supplementary File** for production and characterization), and biochemical assays were subsequently measured 24 h later. Experiments were performed in quadruplicates using cultures derived from three different human fetal brains.

Extracellular LDH Activity as a Measurement for Cytotoxicity

The release of lactate dehydrogenase (LDH) into culture supernatant correlated with the amount of cell death and membrane damage, providing an accurate measure of cellular toxicity. LDH activity was assayed using a standard spectrophotometric technique described by Koh and Choi (1987).

Caspase-3 Activity as a Measurement for Apoptosis

Caspase-3 activity is a well-established apoptotic biomarker. We quantified caspase-3 activity in brain cells using a commercially

available kit (R&D systems) according to the manufacturer's instructions. Briefly, an aliquot of cell homogenate was incubated with the labeled substrate DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroaniline), which is cleaved by the caspase-3 enzyme and releases the chromophore pNA. The levels of pNA were detected using the BMG Fluostar Optima multimode plate reader (NY, USA), at a wavelength of 405 nm.

7′-Dichlorofluorescin Diacetate (DCFDA) Assay for Production of Reactive Oxygen Species

Cells were incubated in 10 μM DCFDA (Sigma) in PBS at 37°C for 30 min as previously described (Chiu et al., 2013). Treatments were added in PBS. After addition of treatments, Fluorescence was measured at 0 min and 90 min with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The initial zero-point readings were subtracted from the 90 min readings as background.

Quantification of 8-Hydroxy-2′-Deoxyguanosine (8-OH-dG) as a Marker for Oxidative DNA Damage

We used 8-Hydroxy-2′-Deoxyguanosine (8-OH-dG) as a biomarker for oxidative DNA on brain cells exposed to different treatments using the commercially available HT 8-OH-dG ELISA II kit (R&D Systems) as guided by the manufacturer's instructions. Briefly, the 8-OH-dG monoclonal antibody binds competitively to the pre-coated 8-OH-dG as well as in the cell homogenate. Antibody bound to 8-OH-dG in the cell homogenate is washed away during washing and only antibody bound to the well was detected using a HRP-conjugate and colorimetric substrate and the BMG Fluostar Optima multimode plate reader (NY, USA).

Measurement of Lipid Peroxidation

Lipid peroxidation level in the brain cell homogenate was quantified using the thiobarbituric acid reactive substances (TBARS) as previously described (Buege and Aust, 1978) and using the BMG Fluostar Optima multimode plate reader (NY, USA).

Measurement of Protein Carbonyl Content

Protein carbonyls were used as a measure of protein oxidation, in brain cell homogenates using DNPH as previously described (Levine et al., 2000) and using the BMG Fluostar Optima multimode plate reader (NY, USA).

Measurement of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) of the Antioxidant Enzymes Activity

Superoxide dismutase (SOD) and GPx activities were quantified using commercially available kits (Cayman chemicals) according to the manufacturer's guidelines. The SOD assay is based on the formation of a formazan dye following exposure to superoxide anions (generated by hypoxanthine xanthine oxidase system) on a tetrazolium salt. The generated superoxide anions are dismutased by SOD leading to a reduction in the formation of formazan dye. The levels of formazan can be quantified at 450 nm was using the BMG Fluostar Optima multimode plate reader (NY, USA). GPx is based on the reduction of hydrogen peroxide by GPx present in the brain cell homogenate using GSH. Glutathione reductase (GR) and NADPH are then used to regenerate GSH. The assay quantified the levels of NADPH at 340 nm using the BMG Fluostar Optima multimode plate reader (NY, USA).

Measurement of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was assessed in brain cell homogenates using the commercially available kit (Cayman total antioxidant assay) in accordance to the manufacturer's guide using the BMG Fluostar Optima multimode plate reader (NY, USA).

Determination of Reduced GSH and Oxidized to Reduced GSH Ratio

The GSH assay method used in this study was adapted from Rahman et al. (2006) and all buffer formulations and assay reagent preparations were based on the original protocol. Briefly, neuronal homogenates extracts were diluted in Potassium phosphate-EDTA (KPE) buffer such that the sample values would fall within the standard curve range. Standards were prepared in matching dilutions of the lysis/extraction buffer in KPE buffer. Afterward, 20 μl of KPE buffer, serving as the blank, along with 20 μl of each of the standards, were dispensed into wells of a single row of a 96-well microplate. The assay's designated standard range was between 0.1 through to 46.0 nM. The plate was loaded into BMG's Fluostar Optima multimode plate reader (NY, USA), controlled by native software (version 2.10 R2). Both pumps were employed to dispense assay reagents, with Pump 1 being used to deliver a solution of equal parts GR and 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB) in KPE. Pump 2 was used to deliver β-NADPH in KPE. One-hundred and twenty microliter of the combined GR and DTNB reagent and 60 μl of β-NADPH reagent was dispensed per well, into wells containing samples/standards. The rate of chromogenic TNB (5-thio-2-nitrobenzoic acid) formation from the non-chromogenic substrate DTNB [5,5′-dithio-bis (2-nitrobenzoic acid)] is directly proportional to the amount of GSH present and can be measured at 412 nm. Oxidized to reduced GSH ratio was determined using GSSG/GSH detection kit (Enzo Diagnostics, New York, NY, USA) as described in the manufacturer's guidelines.

Quantification of a Panel of Inflammatory Cytokines

The levels of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in brain cell homogenates were quantified using specific ELISA kits (Ray Biotech, Peachtree Corners, GA, USA) according to the manufacturer's instructions. These assays use biotinylated antibodies and streptavidin–HRP conjugate and a TMB (3,3′,5,5′-tetramethylbenzidine)-based detection system.

Measurement of Matric Metalloproteinase Activity (MMP-2 and MMP-9)

Matric metalloproteinase (MMP)-2 and MMP-9 activities were quantified using commercially available kits (AnaSpec) according to the manufacturer's guidelines. The MMP-2 and MMP-9 activity assays use a 5-FAM (fluorophore) and QXL520^{TM} (quencher) labeled FRET peptide substrate to quantify MMP-2 and MMP-9 activity in the sample. Cleavage of the FRET peptide by MMP-2 and MMP-9 alters the fluorescence of 5-FAM which can be quantified at excitation/emission of 490 nm/520 nm using the BMG Fluostar Optima multimode plate reader (NY, USA).

Bradford Protein Assay for the Quantification of Total Protein

LDH, caspase 3, SOD and GPx activities, the levels of DCFDA, 8-OH-dG, TBARS, DNPH, GSSG/GSH and several cytokines, and TAC were adjusted for variations in cell number using the Bradford protein assay described by Bradford (1976).

Data Analysis

The results obtained are presented as the means ± the standard error of measurement (SEM). One-way analysis of variance (ANOVA) and *post hoc* Tukey's multiple comparison tests were used to determine statistical significance between treatment groups. Differences between treatment groups were considered significant if p was less than 0.05 ($p < 0.05$).

RESULTS

Characterization of Recombinant Oligomeric Aβ$_{40}$

Recombinant preparations of oligomeric Aβ$_{40}$ were prepared and characterized using atomic force microscopy (AFM). **Supplementary Figure S1A** shows oligomeric Aβ$_{40}$ as evidenced by a homogeneous population of spherical particles averaging 3–5 nm in size. No mature amyloid fibril structures were observed in these preparations.

We further characterized our oligomeric Aβ$_{40}$ preparation using western blotting (**Supplementary Figure S1B**) using the monoclonal antibody 6E10 recognizing residues 1–17 of Aβ. Recombinant oligomeric Aβ$_{40}$ preparations contained large amounts of low molecular weight species corresponding to monomer, dimer, trimer and tetramer, with a smear of Aβ species between 16 kDa and 50 kDa. Similar trends in migration patterns for oligomeric Aβ$_{40}$ have been observed in other laboratories using the same Aβ aggregation method (Dahlgren et al., 2002). However, other laboratories have also reported detection of a larger molecular weight oligomeric Aβ species in their recombinant preparations that range from 40–98 kDa (Garzon and Fahnestock, 2007). Differences in these results may be due to variations in western blotting techniques used from laboratory to laboratory, for example, the specific composition of gels and the running buffer/s used.

Neurotoxic Effect of Oligomeric Aβ$_{40}$ in Isolated Adult Astrocytes Can be Ameliorated Using GGC

Figure 1A shows the effect on neuronal viability, assessed by release of LDH into the media of human primary astrocytes following treatment with 10 nM oligomeric Aβ$_{40}$ for 24 h. Oligomeric Aβ$_{40}$ significantly decreased astrocyte cell viability. Treatment with GGC (200 μM) provided significant protection from exposure to oligomeric Aβ$_{40}$. Treatment of astrocytes with GGC significantly reduced caspase 3 activity (**Figure 1B**), supporting the protective effects of GGC against oligomeric Aβ$_{40}$-mediated apoptosis.

Oligomeric Aβ$_{40}$ Induces Free Radical Damage to DNA, Lipids, and Protein Which Could be Attenuated Using GGC

To confirm that oligomeric Aβ$_{40}$ is cytotoxic to astrocytes *via* induction of oxidative stress, we monitored the effect of oligomeric Aβ$_{40}$ on free radical generation. **Figure 2A** demonstrates that incubation with oligomeric Aβ$_{40}$ can increase reactive oxygen species production using the DCFDA Assay, and which was reduced by treatment with GGC. Co-treatment with GGC ameliorated oxidative DNA damage (**Figure 2B**), lipid peroxidation (**Figure 2C**), and protein carbonyl formation (**Figure 2D**).

Oligomeric Aβ$_{40}$ Depletes Endogenous GSH Levels Which Can be Attenuated Using the GGC

Oligomeric Aβ$_{40}$ decreased the activity of the antioxidant enzymes SOD (**Figure 3A**) and GPx (**Figure 3B**) compared to non-treated cells. Similarly, it reduced the levels of the GSH (**Figure 3C**) and the TAC (**Figure 3D**) in brain cell homogenates and increased the ratio GSSG/GSH (**Figure 3E**). Co-administration with GGC significantly increased the activity of SOD and GPx. Similarly, GGC increased the levels of GSH and TAC and significantly reduced the GSSG/GSH ratio. Therefore, GGC may attenuate oligomeric Aβ$_{40}$-mediated disruption of endogenous antioxidant enzymes and replenish GSH levels in human brain cells.

Oligomeric Aβ$_{40}$ Induces Inflammation Which Is Modulated by GGC

Treating human brain cells with oligomeric Aβ$_{40}$ significantly decreased the level of the anti-inflammatory cytokine IL-10 (**Figure 4A**) and increased the levels of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β compared to non-treated cells (**Figures 4B–D**). GGC effectively modulated the observed oligomeric Aβ$_{40}$-induced changes in the levels of inflammatory cytokines *in vitro*. We observed a significant upregulation in IL-10 levels and significant downregulation of TNF-α, IL-6, and IL-1β in GGC-treated cells, suggestive of the beneficial effects of GGC against oligomeric Aβ$_{40}$-induced neuroinflammatory response.

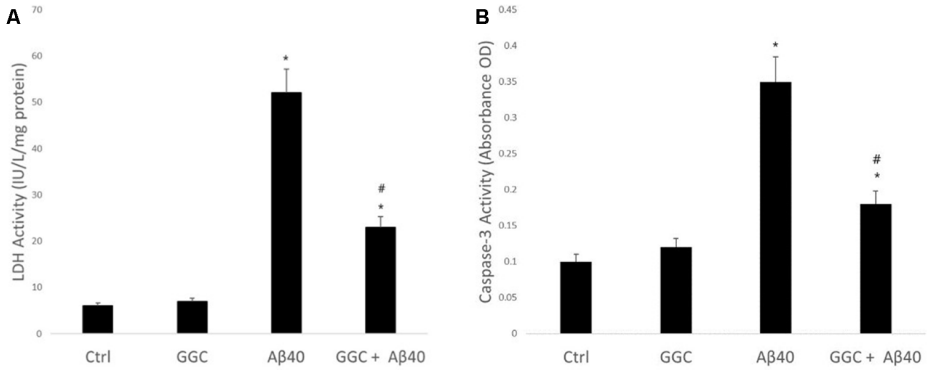

FIGURE 1 | Neurotoxic effect of oligomeric $A\beta_{40}$ in isolated adult astrocytes can be ameliorated using GGC. **(A)** $A\beta_{40}$ significantly decreased astrocyte cell viability. Treatment with GGC (200 mM) provided significant protection from exposure to oligomeric $A\beta_{40}$. **(B)** Treatment of astrocytes with GGC significantly reduced caspase 3 activity. Significance $^{*}p < 0.05$ compared to control non-treated cells. $^{\#}p < 0.05$ compared to control cells treated with oligomeric $A\beta_{40}$.

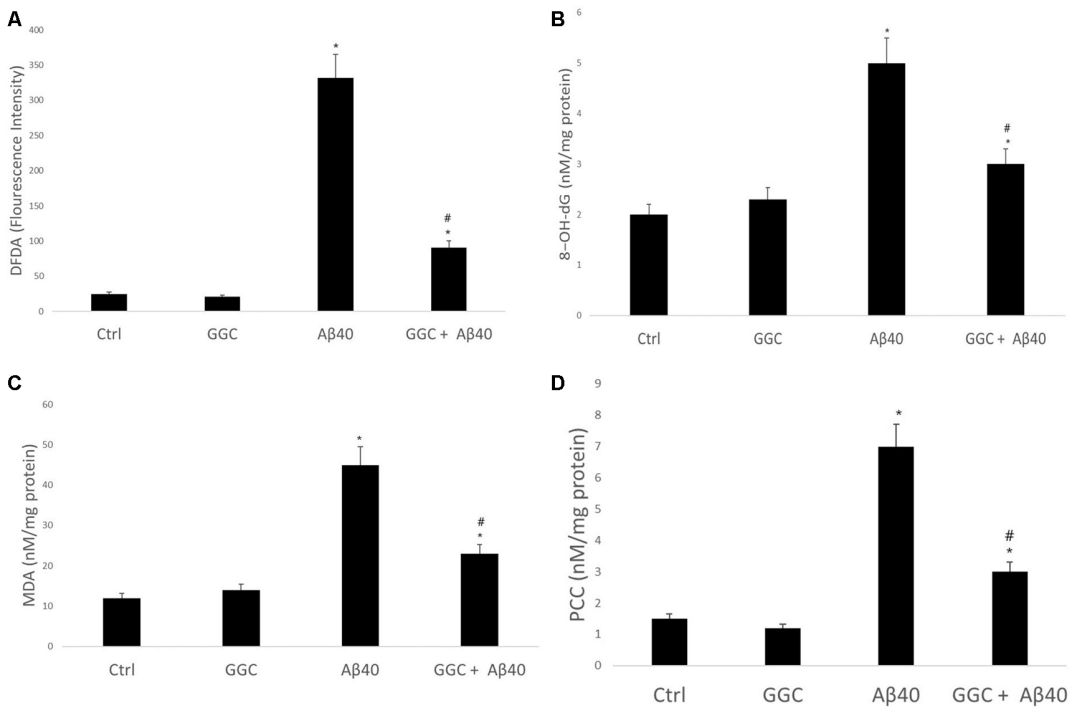

FIGURE 2 | GGC attenuates oligomeric $A\beta_{40}$-mediated oxidative stress production and oxidative damage to DNA, lipids, and protein in isolated adult astrocytes. **(A)** Oligomeric $A\beta_{40}$ increased reactive oxygen species production using the DCFDA Assay, and which was reduced by treatment with GGC. Co-treatment with GGC also ameliorated **(B)** oxidative DNA damage, **(C)** lipid peroxidation, and **(D)** protein carbonyl formation. Significance $^{*}p < 0.05$ compared to control non-treated cells. $^{\#}p < <0.05$ compared to control cells treated with oligomeric $A\beta_{40}$.

GGC Supplementation Reduces Metalloproteinase Activity Induced by Oligomeric $A\beta_{40}$

Treating human brain cells with oligomeric $A\beta_{40}$ significantly increased the activity of MMP-2 (**Figure 5A**) and decreased the activity of MMP-9 compared to non-treated cells (**Figure 5B**). GGC attenuated the effect of oligomeric $A\beta_{40}$-induced changes in MMP-2 and MMP-9 activities *in vitro*. We observed a significant decrease in MMP-2 activity and a significant increase in MMP-9 activity in GGC-treated cells, suggestive of the differential effect of GGC and increased GSH on MMPs.

DISCUSSION

GSH is the most potent antioxidant in the human body. It has multiple antioxidant actions which include direct conjugation

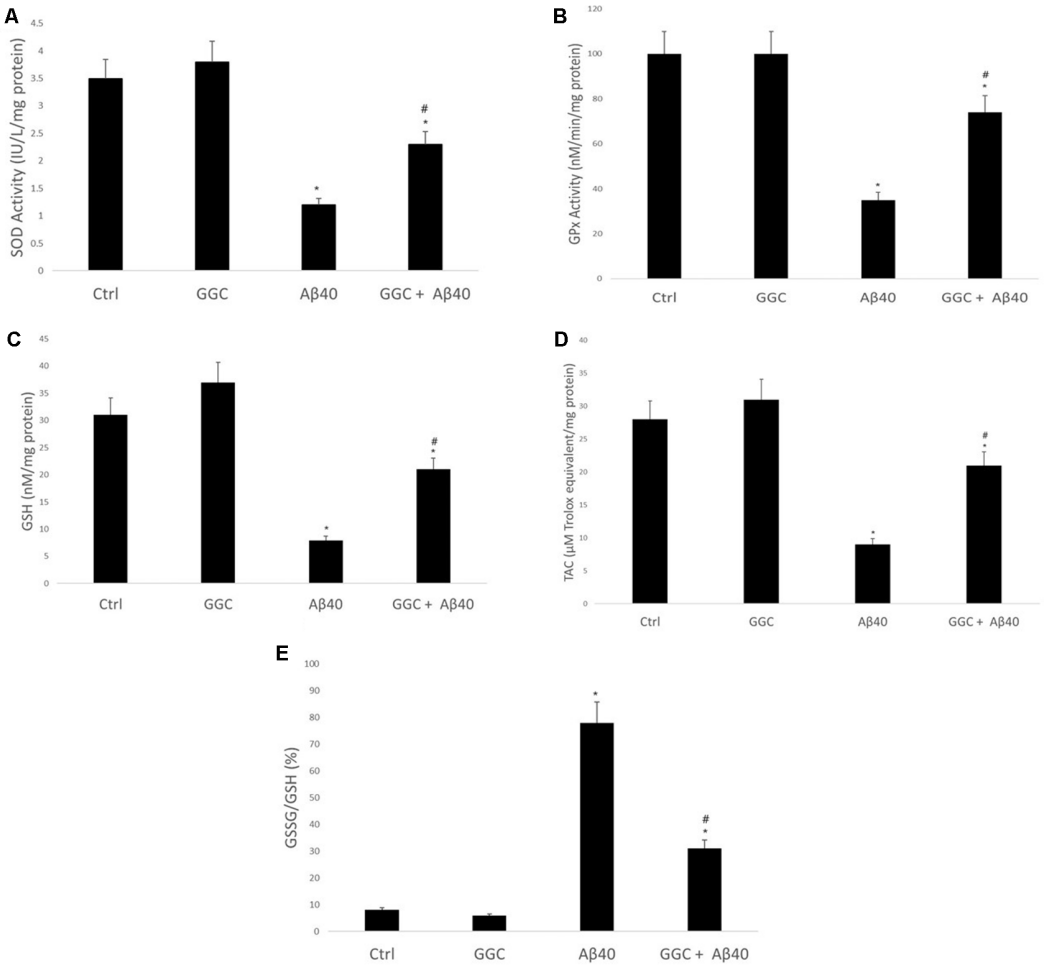

FIGURE 3 | GGC increases **(A)** SOD and **(B)** GPx activities and **(C)** total antioxidant capacity, **(D)** attenuates oligomeric $A\beta_{40}$-mediated GSH depletion, and **(E)** decreases the GSSG/GSH ratio in isolated adult astrocytes. Significance $^{*}p < 0.05$ compared to control non-treated cells. $^{\#}p < 0.05$ compared to control cells treated with oligomeric $A\beta_{40}$.

with free radicals, enzyme-mediated neutralization of free radicals, and the regeneration of other eminent antioxidants such as Vitamin C. Owing to its potent biological availability, replenishing GSH levels following GSH depletion is an important therapeutic target. In this study, we attempted to evaluate whether the GSH precursor, GGC, could increase GSH levels in primary astrocytes and protect primary astrocytes against biomarkers of apoptosis and cell death, oxidative stress, and neuroinflammation when exposed to pathophysiological concentration of oligomeric $A\beta_{40}$.

Aβ is a peptide that is formed following the proteolytic cleavage of the amyloid precursor protein (APP; Murphy and LeVine, 2010). It forms the principal component of extracellular amyloid plaques which are one of the main pathological hallmarks of AD. While several Aβ species with variable lengths (38–43 amino acids) have been identified, $A\beta_{40}$ is thought to be most abundant (80%–90%) followed by $A\beta_{42}$ (5%–10%; Selkoe, 2001). These peptides have been localized in amyloid plaques and can form oligomers and protofibrils, although $A\beta_{42}$ is more hydrophobic and fibrillogenic (Selkoe, 2001). Post-mortem analysis of human brains from AD patients showed that that $A\beta_{40}$ discriminated between AD patients and high pathology controls more readily than $A\beta_{42}$ (Gao et al., 2010). $A\beta_{40}$ oligomers have been shown to induce neurotoxicity and disrupt brain lipid bilayers while other amyloid species do not (Bode et al., 2019). $A\beta_{40}$ is induced by an increase in intracellular Ca^{2+}, and this has led to the hypothesis that the accumulation of intracellular Aβ may occur as an early event in the pathogenesis of AD (Itkin et al., 2011). Therefore, aggregation of intracellular Aβ may be an adaptive response to increases in $[Ca^{2+}]_i$ and may enhance Ca^{2+}-mediated excitotoxicity, which may induce progressive memory loss and cognitive deficits and enhance neuronal cell apoptosis. This can explain why ageing is a major risk factor for AD, since when calcium imbalance is more pronounced with advanced age.

Our data show that addition of $A\beta_{40}$ oligomers increased cytotoxicity in astrocytes after a 24-h incubation. On the

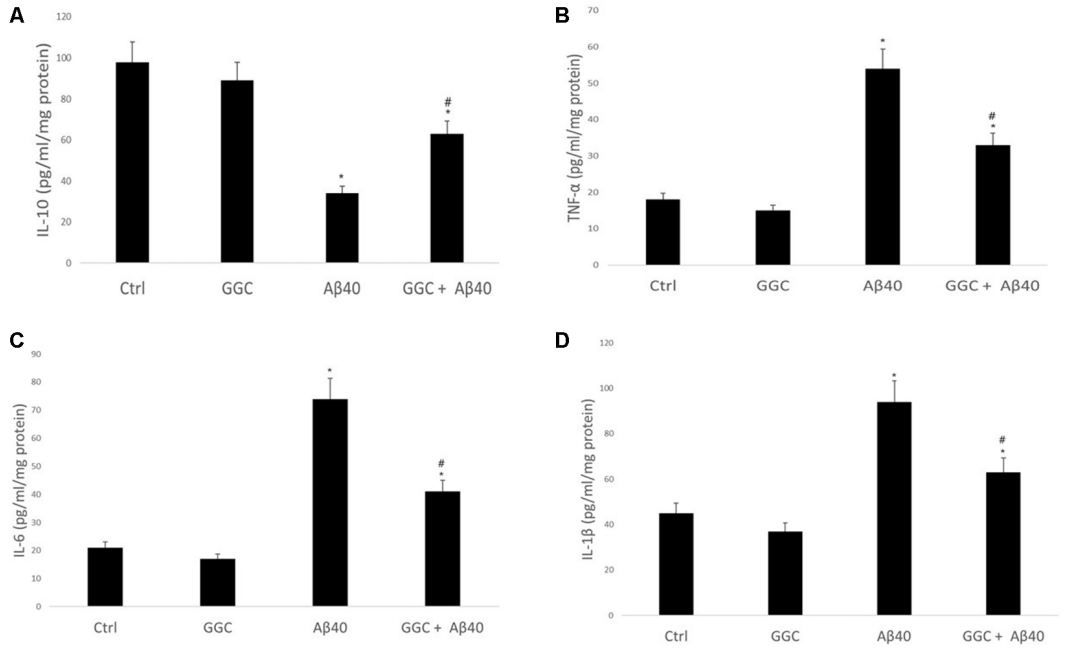

FIGURE 4 | GGC increases **(A)** the levels of the anti-inflammatory cytokine IL-10 and decreases the levels of pro-inflammatory cytokines, **(B)** TNF-α, **(C)** IL-6 and **(D)** IL-β in isolated adult astrocytes. Significance $^{*}p < 0.05$ compared to control non-treated cells. $^{\#}p < 0.05$ compared to control cells treated with oligomeric $A\beta_{40}$.

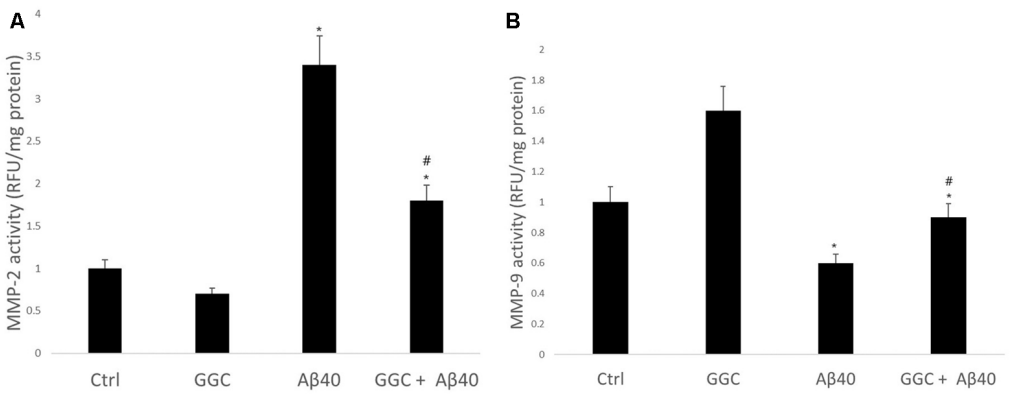

FIGURE 5 | GGC reduces **(A)** MMP-2 and **(B)** MMP-9 activities and attenuates oligomeric $A\beta_{40}$-induction of metalloproteinase activity in isolated adult astrocytes. Significance $^{*}p < 0.05$ compared to control non-treated cells. $^{\#}p < <0.05$ compared to control cells treated with oligomeric $A\beta_{40}$.

contrary, treatment of astrocytes with GGC attenuated cell viability and decreased oligomeric $A\beta_{40}$-mediated oxidative stress and release of pro-inflammatory cytokines *in vitro* after 24 h. Our results suggest that GGC can enter astrocytes and increase GSH levels and reduce the GSSG/GSH ratio, which in turn, attenuates intracellular oxidative stress and protect astrocytes from oligomeric $A\beta_{40}$-mediated oxidative stress and inflammatory response.

MMPs are important proteolytic enzymes necessary for the maintenance of the integrity of the extracellular matrix. Under normal physiological conditions, MMPs are secreted as inactive zymogens. However, under pathological conditions, activated MMPs are associated with increased degradation of ECM components and are involved in ECM re-modeling (Dollery et al., 1995). MMP2 has been reported to be a key player in the disruption of tight junction proteins and enhance blood brain barrier (BBB) permeability (Yang et al., 2007). Consistent with these findings, our data shows that $A\beta_{40}$ oligomers can increase MMP-2 activity in stimulated astrocytes and correlated with increased oxidative stress, inflammation and reduced cell viability. MMP-9 has been shown to degrade fibrillar deposits and is co-localized with vascular amyloid

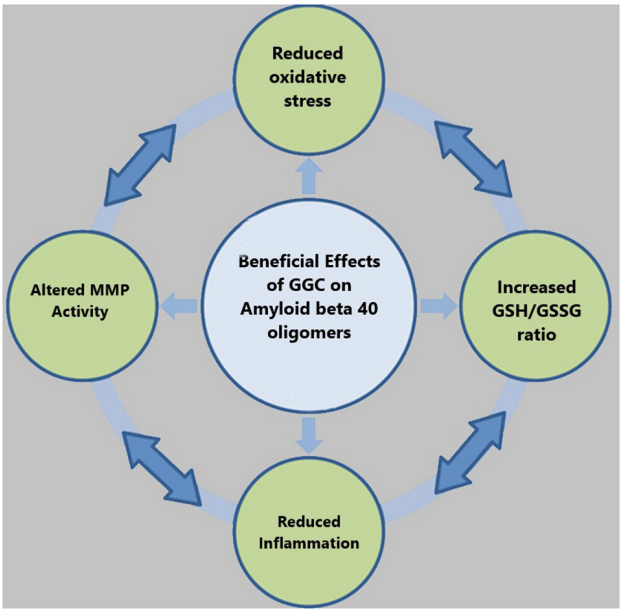

FIGURE 6 | GGC can protect against oligomeric $A\beta_{40}$ toxicity by reducing oxidative stress and increasing GSH levels.

deposits and neurofibrillary tangles (Alvarez-Sabín et al., 2004). However, since only the latent form of MMP-9 has been shown to accumulate in the AD brain, it has been hypothesized that impaired MMP-9 activity may lead to accumulation of abnormal Aβ peptides in amyloid plaques (Lim et al., 1996). With regards to this hypothesis, our data shows that $A\beta_{40}$ oligomers reduce MMP-9 activity in human astrocytes and MMP-9 activity is increased following treatment with GGC. Our results are in line with previous findings showing the direct inhibitory effect of GSH on MMP-2 gelatinolytic capacity, and GSH-mediated increases in MMP-9 gelatinolytic activity *in vitro* (Bogani et al., 2007). The same study reported upregulation of MMP-9 transcription and activity following treatment with GSH (Bogani et al., 2007), and we observed a similar finding in GGC treated cells alone. Therefore, the differential effect of GGC on MMPs is likely to reduce ECM breakdown and promote degradation of Aβ peptide and may be dependent on GSH status.

We additionally found that GGC can increase intracellular GSH levels and lower the GSSG/GSH ratio in astrocytes. Alterations in the levels of either reduced (GSH) or oxidized (GSSG) GSH may affect the function of insulin-degrading enzyme (IDE) which is involved in mediating insulin degradation (Kulas et al., 2019). IDE is also a major regulator of Aβ peptides and reduced IDE activity can reduce Aβ degradation by more than 50% (Farris et al., 2003). One study demonstrated the inhibitory effects of GSSG on IDE activity, potentially due to antagonism of substrate binding. However, GSH enhanced IDE activity non-enzymatically by reducing exposed disulfide bonds and stimulating insulin breakdown (Cordes et al., 2011). Therefore, the intracellular GSH/GSSG ratio may affect the processing and degradation of both insulin and Aβ. Further studies are necessary to confirm whether GGC can increase IDE activity due to increased GSH levels, and may represent an additional mechanism to explain the potential neuroprotective effects of GGC in AD.

GGC is a dipeptide containing cysteine and glutamic acid. Apart from the metal chelating activity of cysteine residue that has been demonstrated in N-acetylcysteine (NAC; Sevgiler et al., 2011), glutamic acid has been reported to chelate redox-active metals. Therefore, the presence of both cysteine and glutamic acid residues in GGC may be likely responsible for its potent antioxidant and anti-inflammatory effects in brain cells against insult oligomeric $A\beta_{40}$. In general, dipeptides have been considered unlikely to be effective as oral drug candidates, since they are prone to hydrolysis by digestive or serum proteases. However, GGC consists of a gamma-glutamyl bond which makes it resistant to hydrolysis by endoproteases (Anderson and Meister, 1983). As well, the importance of GGC as a cofactor for GPx to increase GSH levels is also another mechanism for the protective effects of GGC in human brain cells. The observed immunomodulatory effects of GGC on oligomeric $A\beta_{40}$-induced inflammation may, thus, be also due to chelation of redox-active metals and additional antioxidant mechanisms (**Figure 6**).

The effects of GGC in human astrocytes is relevant for neuroprotection in AD and other neurodegenerative diseases since astrocytes represent the supporting cells in the brain and protect neurons against oxidative stress and enhance neuronal survival during cytotoxic conditions. It has been demonstrated that GSH depleted astrocytes lose their neuroprotective roles, leading to neuronal toxicity and a consequent reduction in cell viability (Pizzurro et al., 2014a,b). Depletion of GSH in astrocytes is also deleterious to astrocytes themselves (Im et al., 2006; Oz et al., 2006). Astrocytes are responsible for the clearance of debris in extracellular space and are required to maintain blood-brain barrier stability (He et al., 2008). The loss of astrocyte function can compromise the function and structure of neurons, culminating in neurodegeneration, and overall brain dysfunction (Schilling et al., 2006). Thus, maintenance of optimal GSH levels is necessary for neuroprotection against oxidative stress and neuroinflammation in astrocytes by maintaining a healthy environment in the CNS.

Strategies aimed at elevating GSH levels have been met with mixed success. The limiting factors include: (1) the inability of GSH to cross the cell membrane *via* direct absorption; (2) feedback inhibitory effects of GSH on glutamate cysteine ligase (GCL) activity (the first biosynthetic enzyme), which is regulated by the availability of cysteine (Jones, 2011); and (3) reduced activity of GCL with advanced age (Liu and Dickinson, 2003). Moreover, several cysteine-based antioxidant trials on AD patients have been unsuccessful (Berk et al., 2013). The therapeutic potential for GGC to increase GSH is related to the second GSH biosynthetic enzyme, glutathione synthetase (GS) which is not regulated by non-allosteric feedback inhibition by GSH. GS catalyzes the addition of glycine to GGC to produce GSH (Zarka and Bridge, 2017). This provides support for the benefits of GGC as an immediate precursor to GSH.

Direct delivery of GCC to the brain has been previously reported to protect against cellular depletion of GSH in the brain using the Buthionine sulfoximine (BSO) model of depletion and increase GSH levels in the pre-perturbed model (Pileblad and Magnusson, 1992; Dringen et al., 1997). It has been reported that the cytosolic concentrations of GGC are close to 7 μM, and passive uptake of intact GGC can be directed into cells. Reduced GSH levels due to impaired GCL activity is unlikely to be replenished by other GSH precursors such as NAC or other cysteine prodrugs. Supplementation with GGC is likely to increase GS activity and attenuate GSH depletion.

Previous studies such as those by Dringen et al. (1997) relied on limiting or sub-physiological conditions to examine the effects of GGC on increases in GS activity in astrocyte-rich primary cultures. However, these do not accurately represent healthy pre-pathological conditions and are unlikely to add support to the ability of GGC to bypass feedback inhibition of GCL. Le et al. (2011) showed that 2-h GGC treatment at 100 or 500 μM, following 4-h exposure to H_2O_2, could not significantly replenish GSH levels or increase GSH levels above homeostatic levels in murine neurons and astrocyte cell cultures. However, in that study, the neuronal and glial cells were co-treated with cytotoxic concentrations of H_2O_2, and therefore it is unlikely that GGC could produce beneficial effects on GSH in this condition. As well, that study could be improved by adding an additional control condition which was exposed to GGC in the absence of H_2O_2 insult.

Recently, our group showed that orally dosed GGC (2 and 4 g) is bioavailable and can increase intracellular GSH levels above homeostasis in lymphocytes of healthy, non-fasting human subjects with no adverse effects (Zarka and Bridge, 2017). This suggests that GGC may increase GSH levels in the clinic with potential as adjunct therapy for the treatment of disorders associated with acute and/or chronic GSH depletion. Previous preclinical studies have demonstrated little or no toxicity following administration of GGC (Joshi et al., 2007; Espinosa-Diez et al., 2015; Zhang et al., 2015; Ding et al., 2016; Henderson et al., 2016; Salama et al., 2016). The present study set out for the first time to evaluate GGC as a GSH-elevating strategy in primary human astrocytes, starting from homeostatic levels, with promising data.

In conclusion, this study has shown that GGC can decrease apoptosis, oxidative stress and inflammation in human astrocytes and protect astrocytes from oligomeric $A\beta_{40}$-mediated cytotoxicity cellular GSH depletion. The beneficial effects of GGC on human brain cells may prevent against neurodegeneration by increasing the availability of GSH precursors, which would contribute to the neuroprotective effects of GGC in AD.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

The approval for this study was obtained from the Human Research Ethics Committee of the University of New South Wales (human brain tissue reference number HC12563).

AUTHOR CONTRIBUTIONS

NB, DC, and PS formulated the present hypothesis. NB was responsible for writing the report. NB, MZ, B-EJ, JW, TJ, and WB performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00177/full#supplementary-material

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REVIEW
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Lifestyle Modifications and Nutritional Interventions in Aging-Associated Cognitive Decline and Alzheimer's Disease

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Alzheimer's disease (AD) is a type of incurable neurodegenerative disease that is characterized by the accumulation of amyloid-β (Aβ; plaques) and tau hyperphosphorylation as neurofibrillary tangles (NFTs) in the brain followed by neuronal death, cognitive decline, and memory loss. The high prevalence of AD in the developed world has become a major public health challenge associated with social and economic burdens on individuals and society. Due to there being limited options for early diagnosis and determining the exact pathophysiology of AD, finding effective therapeutic strategies has become a great challenge. Several possible risk factors associated with AD pathology have been identified; however, their roles are still inconclusive. Recent clinical trials of the drugs targeting Aβ and tau have failed to find a cure for the AD pathology. Therefore, effective preventive strategies should be followed to reduce the exponential increase in the prevalence of cognitive decline and dementia, especially AD. Although the search for new therapeutic targets is a great challenge for the scientific community, the roles of lifestyle interventions and nutraceuticals in the prevention of many metabolic and neurodegenerative diseases are highly appreciated in the literature. In this article, we summarize the molecular mechanisms involved in AD pathology and the possible ameliorative action of lifestyle and nutritional interventions including diet, exercise, Calorie restriction (CR), and various bioactive compounds on cognitive decline and dementia. This article will provide insights into the role of non-pharmacologic interventions in the modulation of AD pathology, which may offer the benefit of improving quality of life by reducing cognitive decline and incident AD.

Keywords: Alzheimer's disease, oxidative stress, inflammation, diet, exercise, lifestyle, nutraceuticals, antioxidants

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INTRODUCTION

Due to improved healthcare infrastructures and early diagnosis of diseases, human life expectancy is increasing globally (Oeppen and Vaupel, 2002). However, the burden of age-associated diseases will also increase exponentially in the coming decades (Beard et al., 2016). Aging is a process that is regulated by various genetic and molecular mechanisms. Dementia is one of the major causes of disability in the aging population. Alzheimer's disease (AD), the most dominant form of dementia, accounting for 60–80% of dementia cases, is a multifactorial neurodegenerative disease characterized by the accumulation of amyloid-β (Aβ; plaques) outside neurons and the hyperphosphorylation of tau protein and neurofibrillary tangles (NFTs) inside the neurons in the brain, which lead to cognitive deficit, memory loss, and then neuron death. With the aging of the population, the burden of age-related neurodegenerative disorders is increasing at an exponential rate all over the world (Prince et al., 2015). Multiple risk factors including age, education, socioeconomic status, family history, gene mutations, oxidative damage, neuroinflammation, et cetera are involved in the pathophysiology of AD. Currently, pharmacologic drugs targeting Aβ and tau proteins are unsuccessful in clinical trials, and there is no treatment available for the cure of AD to date. Due to the failure of pharmacologic treatments for AD, non-pharmacological interventions have gained more attention for the prevention of this disease. Many common molecular mechanisms are shared among the metabolic and neurodegenerative disorders, which include mitochondrial dysfunction, oxidative stress, inflammatory pathways, and calcium homeostasis. There are many preventive strategies that target these pathways and are found to be very effective in limiting the burden of metabolic diseases such as diabetes, obesity, cardiovascular diseases (CVDs), and cancer. In this review article, we focus on the molecular mechanisms involved in AD and the lifestyle and nutritional interventions, such as physical exercise, a Mediterranean diet (MD), nutraceuticals, and bioactive compounds, as preventive strategies for the development of AD in the elderly population. These interventions for the prevention or delay of the onset of dementia could have significant effects on individuals, society, and healthcare providers.

PREVALENCE AND ECONOMIC BURDEN OF ALZHEIMER'S DISEASE

Current demographic trends indicate a significant increase in older people (above 65 years) in the world's population, posing a major risk for the development of dementia. A previous study indicates that about 6% of the world population is suffering from dementia (Prince et al., 2013). Recent estimates by the Alzheimer's Association reveal that globally, 55 million people have dementia and every year, more than 10 million people develop this deadly disease. This number is predicted to increase by 88 million in the year 2050 (Alzheimer's Association, 2019). This disease is more prevalent in Americans. Currently, 5.8 million Americans of all ages were living with Alzheimer's dementia in 2019, and this number is projected to increase by three folds by the year 2050. About 5.6 million people are above 65 years old. Every 10th American aged above 65 years is suffering from AD. This increase in the incidence and prevalence of AD in the elderly population has become a major public health challenge associated with an economic burden on individuals, society, caregivers, and federal governments (Hurd et al., 2013). **Figure 1** shows the annual expenditure, including for health care, long-term care, and hospice care for people with dementia. Recent estimates predict that there will be a heavy increase in the overall maintenance cost of AD, which is expected to increase from $290 billion in 2019 to more than $1.1 trillion in 2050 (Alzheimer's Association, 2019). However, reducing the potentially modifiable risk determinants, including educational status, cigarette smoking, sedentary lifestyle, depression, hypertension, diabetes, dyslipidemia, and obesity, may help in the prevention of about 500,000 cases of dementia in the United States (GBD 2015 Neurological Disorders Collaborator Group, 2017).

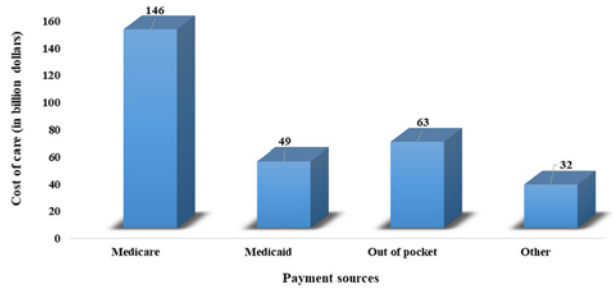

FIGURE 1 | Annual expenditure, including health care, long-term care, and hospice care for people with dementia.

MOLECULAR MECHANISMS INVOLVED IN ALZHEIMER'S DISEASE PATHOLOGY

Amyloid β Plaques and Tau Hyperphosphorylation

Two major proteins in the brain are involved in the pathophysiology of AD, i.e., the Aβ and tau proteins. The Aβ protein, consisting of 39–43 amino acid residues, is produced intracellularly in the brain. A disparity between the accumulation and clearance of Aβ leads to plaque formation in the brain. It plays a vital role in the progress of AD pathology and cognitive impairment (Hardy, 1997; George-Hyslop and Rossor, 2001). Genetic mutations in the genes encoding Aβ, Aβ precursor protein (AβPP), and Presenilins (PS1 and PS2) lead to abnormal Aβ aggregation in the brain. In AD, the build-up of amyloid fibrils as amyloid plaques or senile plaques in the extracellular region of brain cells is responsible for synaptic damage, neuronal dysfunction, and inflammatory responses (Lesné et al., 2013). Tau protein, a family of natively unfolded microtubule-associated proteins, is located on chromosome 17q21 and plays an important role in microtubule assembly and stabilization. In AD pathology, the intense hyperphosphorylation of tau

protein causes the formation of NFTs, leading to microtubule disassembly and neuronal loss in the area of the brain associated with memory and learning centers (Kolarova et al., 2012).

Mitochondrial Dysfunctioning

Mitochondria are double-membrane, intracellular organelles present in the cells and are known to play a vital role by metabolizing nutrients. They are also known as the powerhouse of the cell or "energy currency," as they generate adenosine triphosphate (ATP). Several studies have implicated mitochondrial dysfunction as a major pathologic condition involved in neurodegenerative diseases. Mitochondrial dysfunction involves alterations in the processes of mitochondrial biogenesis and dynamics, which leads to many pathologic conditions. In mitochondrial biogenesis, the number and size of mitochondria increases, which is controlled by peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), involving several transcription factors and other proteins including nuclear respiratory factors (NRF-1 and NRF-2), uncoupling proteins (UCP2), transcription factor A (Tfam), PPARs, thyroid hormone, glucocorticoid, estrogen, and estrogen-related receptors (ERR) α and γ (Hock and Kralli, 2009). AMP-activated protein kinase (AMPK) also contributes to the regulation of intracellular energy metabolism (Reznick and Shulman, 2006; Bhatti et al., 2017a). **Figure 2** shows the regulation process of mitochondrial biogenesis by various transcription factors and other proteins.

Mitochondrial dynamics is a process by which mitochondria maintain their shape, structure, and functions by continuously going through the fission and fusion process (Chan, 2006; Westermann, 2010; Archer, 2013; Roy et al., 2015). In mitochondrial fusion, three GTPase genes, including Mitofusin 1 and 2 and optic atrophy1 (Opa1), regulate this process, whereas the mitochondrial fission is controlled by two GTPase genes, Fis1 and Drp1. Impaired mitochondrial biogenesis and dynamics lead to disturbed normal functioning of mitochondria, resulting in diminished energy generation in the cells. It is evident from previous studies that impaired mitochondrial dynamics plays a vital role in aging and aging-associated metabolic and neurodegenerative diseases (Reddy, 2011). During mitochondrial biogenesis, some defective mitochondria that are formed are then removed by a process called mitophagy. The defective mitochondria fuse with the lysosomes and are removed by the autophagy-lysosome system (Ding and Yin, 2012). Aging contributes to the accumulation of defective mitochondria, oxidative imbalance, and apoptosis by impairing mitophagy (Chistiakov et al., 2014). Modifications in mitochondrial biogenesis and dynamics also lead to the overproduction of reactive oxygen species (ROS) in the cells, ultimately causing oxidative damage. Autophagy is a lysosome-mediated degradative pathway that facilitates the elimination of defective organelles and recycles various cellular components, including lipids and proteins (Shintani and Klionsky, 2004). Impairment in autophagy may lead to the accumulation of Aβ protein in disease conditions. Autophagy deficit may contribute to age-associated neurodegenerative diseases, including AD (Martinez-Vicente, 2015; Zare-Shahabadi et al., 2015). Emerging strands of evidence indicate that the disruption in the mammalian target of rapamycin (mTOR) signaling pathway impacts multiple cellular functions, including autophagy, glucose metabolism, cell growth, and mitochondrial functions, that are central in aging and neurodegenerative

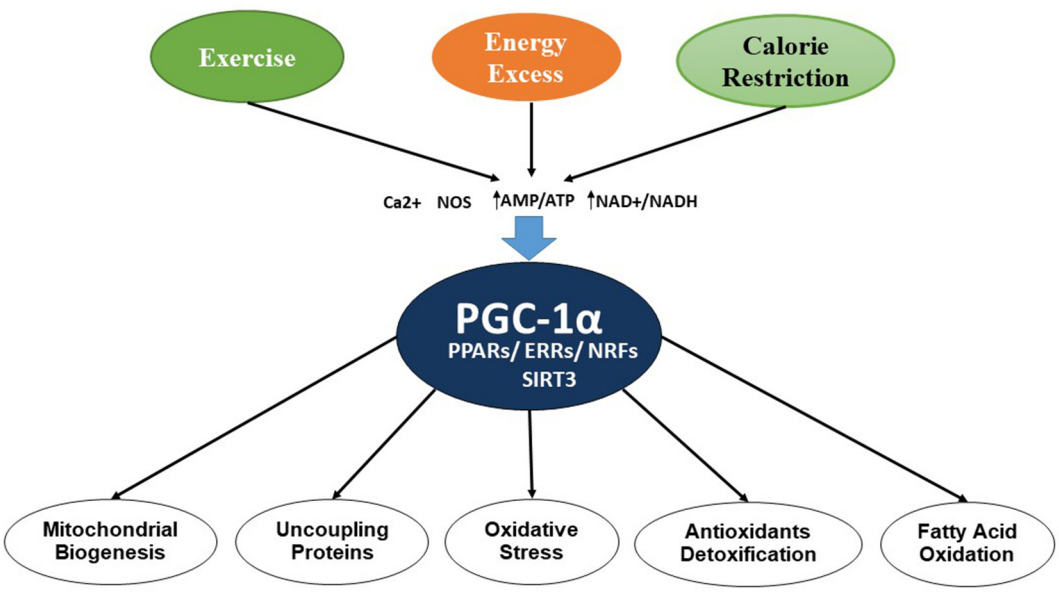

FIGURE 2 | Control of mitochondrial biogenesis by transcription factors and PGC-1α.

diseases (Perluigi et al., 2015). This compelling evidence indicates that targeting mTOR in the brain might be another promising strategy that could enable drug discovery for AD. The dysregulation of the PI3K/AKT/mTOR pathway and autophagy defects in the brains of AD patients might be targeted for the development of new drugs. Reddy and Oliver (2019) recently demonstrated that the accumulation of Aβ and phosphorylated tau induces defective autophagy and mitophagy in AD.

With advanced age, the oxidative damage induced by excessive generation of free radicals reduces antioxidant capacity, and proinflammatory reactions lead to the aging-related pathologic conditions. The brain is very much affected by these oxidative biomarkers. Moreover, the brain normally has less oxidant capacity than other organs. In dementia, the accumulation of neurotoxic peptides such as Aβ and tau might damage the brain tissues (Kapogiannis and Mattson, 2011; Mao and Reddy, 2011). Mitochondria play a vital role in several metabolic processes. The modifications in the mitochondrial structure and function may lead to several age-associated neurodegenerative diseases (Reddy, 2006, 2011; Roy et al., 2015). The generation of various ROS and their scavenging is a routine function that takes place in the mitochondria. An imbalance between the generation of free radicals in the cells and the ability to detoxify is called oxidative stress. In the Kreb's cycle, the electrons are contributed by NADH and $FADH_2$. These electrons are then transferred through the electron transport chain (ETC), which generates electrochemical gradient across the inner mitochondrial membrane and then produces energy in the form of ATP (Andreyev et al., 2005). **Figure 3** shows the process of the generation of free radicals and ATP biosynthesis in the cell. However, this process also leads to the excessive generation of several reactive species such as superoxide anion ($^{\bullet}O_2$), hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO), and reactive nitrogen species (Dröge, 2002; Valko et al., 2006). The overproduction of these ROS may damage proteins, lipids, and DNA (Beckman and Ames, 1999), which disrupts ATP biosynthesis and other functions in mitochondria (Dröge, 2002; Murphy, 2009). Cells tend to neutralize the oxidative damage induced by the overproduction of ROS either by enzymatic or non-enzymatic mechanisms. The main enzymes known to detoxify ROS are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx). On the other hand, there are many non-enzymatic mechanisms that protect the cells against oxidative damage, including glutathione (GSH), vitamins E and C, carotenoids, polyphenols, and flavonoids.

Neuroinflammation

AD pathology is not restricted to the aggregation of misfolded amyloid or tau proteins; some immunological mechanisms are also involved in the brain. Neuroinflammation induced by these misfolded proteins is another key hallmark of AD and might be targeted as a therapeutic strategy along with peptides (Heneka et al., 2015). These misfolded proteins, including Aβ plaques and NFTs in the brain, initiate innate immune responses by interacting with toll-like receptors (TLRs) and CD4 cells. There is substantial evidence for neuroinflammation in the early stages of AD development (Yasuno et al., 2008). Recent studies also established the role of variants of many immune receptor genes, including TREM2 and CD33, in the pathogenesis of AD (Griciuc et al., 2013; Guerreiro et al., 2013; Jonsson et al., 2013). Excesses of free radicals, NO, cytokines, and some proteolytic enzymes may be responsible factors that are associated with neuroinflammation and may promulgate neuronal death (Cherry et al., 2014; Yuste et al., 2015). All of these measures are crucial in age-associated cognitive decline and AD pathology.

Epigenetic Control of Neurodegenerative Diseases

The term epigenetics refers to mitotically and meiotically heritable changes in gene expression in response to environmental stimuli, including stress, diet, or exposure to adverse environmental factors, without altering DNA sequences (Babenko et al., 2012; Griñan-Ferré et al., 2016). The main epigenetic mechanisms are DNA methylation, histone post-translational modifications, and the regulation of gene expression mediated by noncoding RNA molecules (Moore et al., 2013; Holoch and Moazed, 2015; Hwang et al., 2017). DNA methylation is a well-known epigenetic mechanism that involves the addition of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine with the help of an enzyme, a DNA methyltransferase. DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA (Moore et al., 2013). Histones are the most abundant proteins associated with DNA and aggregate with each other, forming the histone octamer around which DNA is wrapped to create the nucleosome (Bannister and Kouzarides, 2011). The N-terminal tails of histones may undergo several post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ADP ribosylation. These changes influence the chromatin structure, facilitating or inhibiting gene transcription (Bannister and Kouzarides, 2011). In addition to DNA methylation and histone modifications, the regulation of gene expression mediated by noncoding RNA molecules occurs in many tissues (Peschansky and Wahlestedt, 2014; Holoch and Moazed, 2015).

Global DNA modification studies have highlighted a potential role for epigenetic mechanisms in the complex etiology of various neurodegenerative diseases, particularly AD (Bradley-Whitman and Lovell, 2013; Coppieters et al., 2014; Roubroeks et al., 2017). Besides the nuclear DNA, there is growing evidence that the mitochondrial DNA (mtDNA) could be controlled by epigenetic mechanisms (Hroudová et al., 2014; Blanch et al., 2016; Stoccoro et al., 2017). Several studies have demonstrated the impact of epigenetic modifications on the pathogenesis of neurodegenerative diseases (Urdinguio et al., 2009; Gruber, 2011; Gangisetty and Murugan, 2016; Bassi et al., 2017; Smith and Lunnon, 2017; Berson et al., 2018; Gangisetty et al., 2018; Lardenoije et al., 2018; Lascano et al., 2018; Qazi et al., 2018; Stoccoro and Coppedè, 2018). There

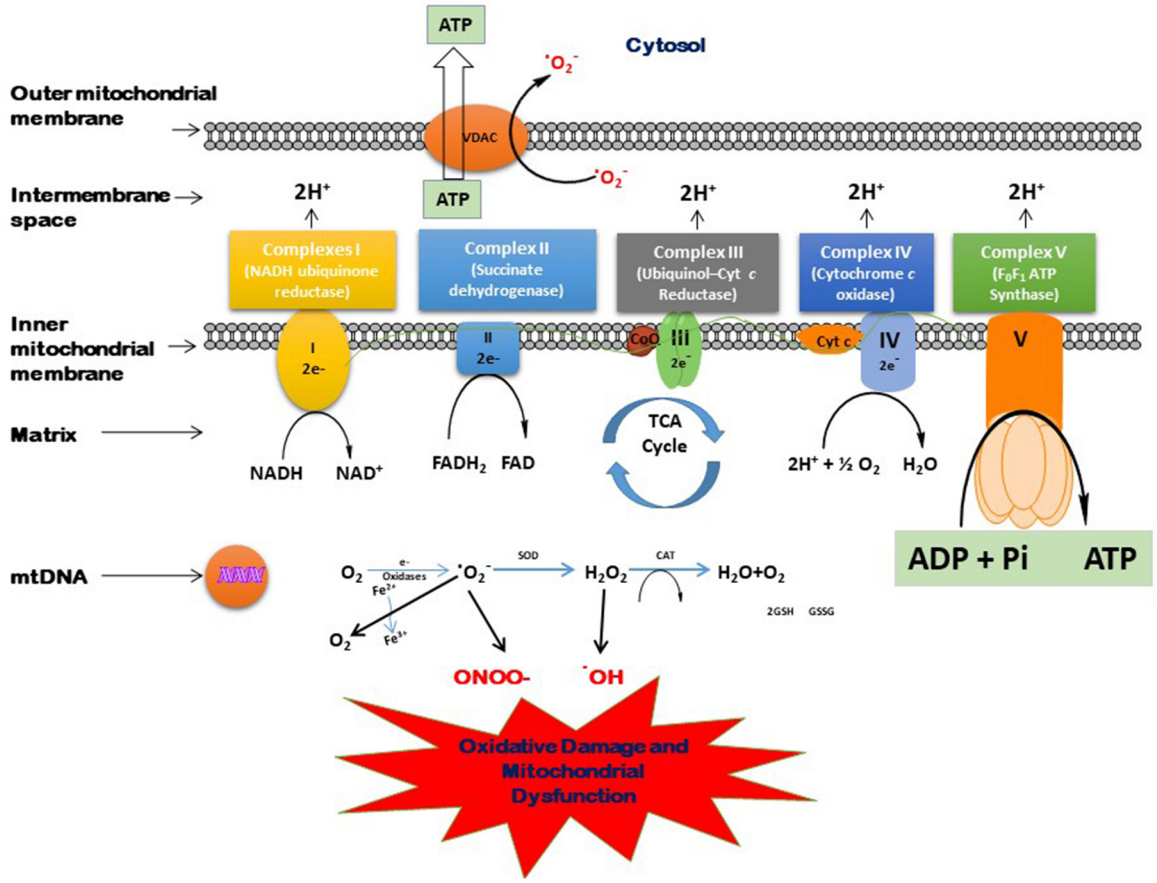

FIGURE 3 | Process of free radical generation and adenosine triphosphate (ATP) biosynthesis in the cell.

is a growing body of evidence suggesting that epigenetic mechanisms mediate the risk for AD. Intense research in experimental models suggests that molecular interventions for modulating epigenetic mechanisms might have therapeutic applications to promote cognitive maintenance to an advanced age (Griñan-Ferré et al., 2016).

Modifiable Risk Factors

Emerging evidence suggests that traditional cardiometabolic risk factors such as a sedentary lifestyle, central obesity, dyslipidemia, insulin resistance, hypertension, diabetes, and CVDs are associated with the progress of cognitive decline and AD (Cholerton et al., 2013; de la Torre, 2013; Chen et al., 2014; Geijselaers et al., 2015; Xu et al., 2015; Tamarai et al., 2019). Conversely, Calorie restriction (CR), antioxidant-rich dietary components, and certain dietary patterns may limit the progress of metabolic and neurodegenerative diseases (Everitt et al., 2006; Calder et al., 2011). The molecular mechanisms linking these modifiable factors are already discussed in our previous study (Bhatti et al., 2017b). **Figure 4** shows various modifiable risk determinants affecting various molecular mechanisms in AD pathology, as demonstrated in a previous study (Chakrabarti et al., 2015).

PHARMACOLOGIC TREATMENTS FOR ALZHEIMER'S DISEASE

Many experimental treatments currently undergoing clinical trials are targeting the molecular mechanisms of AD, including Aβ plaques, tau hyperphosphorylation, oxidative damage, mitochondrial dysfunction, neurotransmission, calcium homeostasis, cell signaling, and anti-inflammatory pathways (Arvanitakis et al., 2008; Leoutsakos et al., 2012; Latta et al., 2015; Bhatti et al., 2017a; Hsu and Marshall, 2017). However, effective pharmacologic treatment strategies for cognitive decline, mild cognitive impairment (MCI), or dementia are not available to date. The accumulation of Aβ peptide and tau hyperphosphorylation are the major hallmarks of AD (Reddy and Oliver, 2019). Several clinical trials of the drugs targeting Aβ peptide and tau hyperphosphorylation failed to demonstrate any positive result. A recent systematic review of 51 unique trials from January 2009 to July 2017 rated as having low to moderate risk of bias established that the currently available drugs for

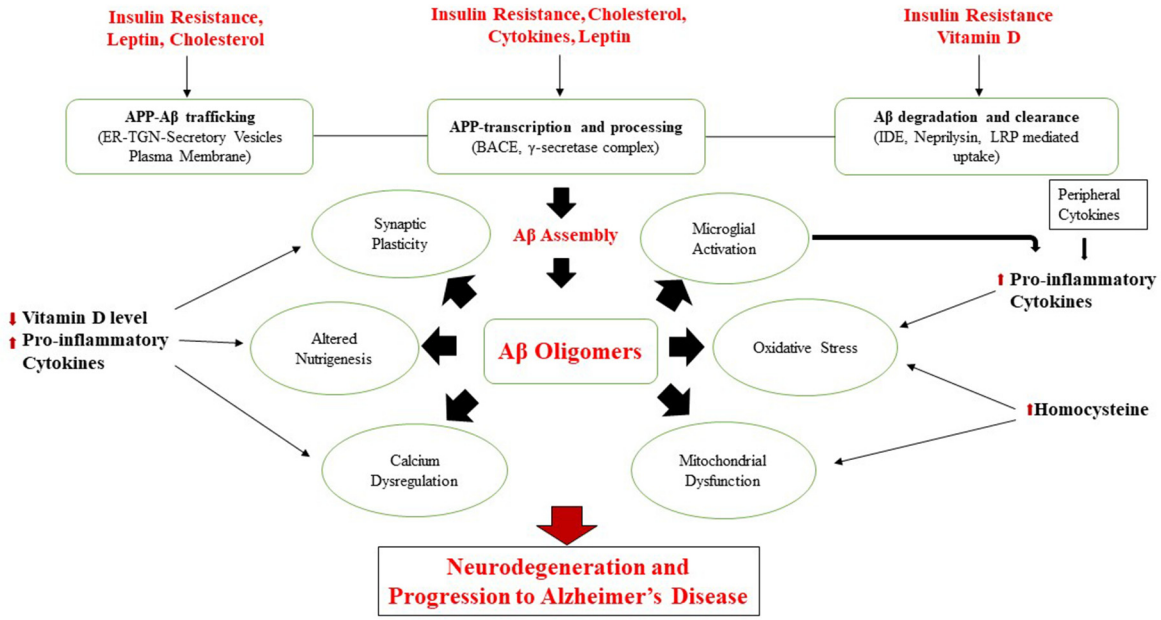

FIGURE 4 | Various modifiable risk determinants in Alzheimer's disease (AD) pathology.

dementia, hypertension, and diabetes, that is, anti-inflammatory medications such as nonsteroidal anti-inflammatory drugs, statins, and estrogen/progestin agents, neither improved nor slowed cognitive decline in persons with normal cognition or with MCI (Fink et al., 2018). This evidence shows the limited scope of these pharmacologic management approaches for cognitive protection in individuals with normal cognition or MCI. Recent studies of clinical trials focusing on cognitive training (11 trials) and physical exercise (16 trials) did not show satisfactory results for the prevention of cognitive decline or dementia (Brasure et al., 2018; Butler et al., 2018). Interestingly, a randomized controlled trial involving the multidomain intervention of exercise, diet, and cognitive training prevented cognitive decline in at-risk older people (Ngandu et al., 2015).

NON-PHARMACOLOGIC INTERVENTIONS IN AD PATHOLOGY

Since no effective pharmacological treatment is available to cure dementia, a greater emphasis has been placed on the implementation of non-pharmacological interventions that may prevent AD or reduce the escalation of AD burden. It is evident from various animal and human observational studies that non-pharmacologic interventions including physical exercise, CR, antioxidant supplements, diet, nutraceuticals, and several plant-based bioactive compounds are effective in reducing the modifiable risk factors such as obesity, diabetes, CVDs, cancer, etc. Many dietary interventions are known to improve insulin sensitivity, which further reduces inflammation and improves cognitive functions (Bayer-Carter et al., 2011; Kelly et al., 2011). Interventions that target modifiable risk factors for AD hold promise for reducing the incidence of AD (Xu et al., 2015). The lifestyle interventions appear to reduce the morbidity and mortality in aging populations by modulating various molecular mechanisms and might be promising non-therapeutic measures for various metabolic and aging-associated diseases (Norton et al., 2014). Recent studies suggest that elevated incidence and prevalence of cognitive decline and AD might be reduced through effective strategies targeting various cardiometabolic risk factors, including sedentary lifestyle, smoking, midlife hypertension, midlife obesity, and diabetes (Norton et al., 2014). Thus, lifestyle and nutritional intervention may be effective primary prevention strategies for AD. The possible mechanisms mediating the impact of lifestyle and nutritional interventions on cognitive decline and AD are discussed here.

DIETARY INTERVENTIONS

Healthy nutritive food components, rich in their antioxidant and anti-inflammatory properties, are known to regulate the immune system and may modify the neuroinflammatory events involved in the progression of cognitive impairment and AD (McGrattan et al., 2019). These nutraceuticals and dietary patterns may constitute promising approaches in the prevention of cognitive decline or delaying the progression to AD (Canevelli et al., 2016). Several dietary components, such as omega-3 fatty acids, nutraceuticals, minerals, micronutrients, and vitamins have been examined for their roles in health and disease (Wilson et al., 2017). These dietary interventions are known to play an ameliorative role in the pathophysiology of diabetes, obesity, CVDs, and cancer, etc. The dietary interventions modulate the molecular mechanisms, including Aβ formation, tau hyperphosphorylation, oxidative stress, and epigenetic controls, in age-associated neurodegenerative diseases. Diet

can modify the epigenetic mechanisms by regulating DNA methylation, acetylation, histone modifications, and changes in miRNA expression, thereby influencing the expression of particular genes responsible for epigenetic alterations (Park et al., 2012; Abdul et al., 2017).

Polyunsaturated Fatty Acids (PUFAs)

Lipids are an essential component of the brain, wherein about one-third are essential Polyunsaturated fatty acids (PUFAs; Benatti et al., 2004). They constitute vital components of neuronal cell membranes and are involved in membrane fluidity, allowing for optimal communication between cells, cell signaling, and neuroprotection (Bazan, 2005). Essential PUFAs play a critical role in brain development and functions, with antioxidant, anti-excitotoxic, and anti-inflammatory activities. Abnormalities in PUFA status have been implicated in neuropsychiatric health and diseases, including AD (Liu et al., 2015). Many studies involving omega-3 fatty acids concerning cognitive decline have been carried out, and they show conflicting results. The long-chain omega-3 PUFAs have been shown to be involved in lowering the risk of cognitive impairment in individuals without dementia (Fotuhi et al., 2009). However, the results of other clinical trials were less conclusive. Thomas et al. (2015) recently summarized the findings of controlled studies carried out over the past 10 years and suggested that omega-3 fatty acid supplementation is advantageous only in the initial stages of cognitive decline. Another study demonstrated that fish intake ($\geq$100 g/week) might slow the progress of cognitive decline in the Chinese population (>65 years; Qin et al., 2014). Another randomized clinical trial involving supplementation of omega-3 PUFA to participants aged 60 years without dementia or cognitive impairment showed no significant improvement in cognitive function (Sydenham et al., 2012). Recently, a randomized, placebo-controlled trial reported that long-term use of omega 3 PUFA supplementation with or without multidomain intervention had no significant impact on the cognitive decline over 3 years (Andrieu et al., 2017).

Curcumin

Curcumin is isolated from the rhizome of *Curcuma longa*, produced mainly in India and China (Ammon et al., 1992). It is the principal active compound of turmeric, an Asian spice, and is known to play a key role in disease prevention through the modulation of various biochemical pathways (Prasad et al., 2014; Kunnumakkara et al., 2017). Turmeric powder is used as a traditional medicine against many conditions because of its antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer activities (Sikora et al., 2010; Rahmani et al., 2018). Frequent use of curcumin in curry may be associated with better cognitive performance and low prevalence of AD in elderly Indian populations compared with the US population (Ganguli et al., 2000; Ng et al., 2006). Recent studies have established that curcumin plays a protective role against Aβ in AD due to its potent antioxidant, anti-inflammatory, and neuroprotective actions (Sundaram et al., 2017; Reddy et al., 2018). A randomized, placebo-controlled, double-blind, clinical trial of curcumin (1–4 g/day) in 34 AD patients shows no significant effect (Baum et al., 2008). Similarly, another randomized clinical trial of Curcuminoids (2 or 4 g/day) in 36 patients with dementia did not show any significant effect (Ringman et al., 2012). Curcumin significantly downregulated the expression of class I HDACs (HDAC1, HDAC3, and HDAC8) and upregulated the acetylated histone H4 levels in Raji cells, thereby modulating the epigenetic control (Liu et al., 2005). Curcumin has been shown to inhibit certain epigenetic enzymes (Reuter et al., 2011; Vahid et al., 2015). The results of several studies indicated that although curcumin has very strong neuroprotective properties, its bioavailability needs to be improved for future therapeutic strategies against neurodegenerative diseases.

Flavonoids

Flavonoids are natural compounds with a polyphenolic structure and are commonly found in fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Panche et al., 2016). According to their chemical composition, flavonoids are categorized into various subclasses such as flavonols, flavones, flavanones, flavanols, anthocyanins, isoflavones, chalcones, and dihydrochalcones. Several studies have suggested that flavonoids display strong antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Pietta, 2000; Panche et al., 2016). Due to these properties, the flavonoids play a preventive role in the pathology of cancer, Alzheimer's, and CVDs (Benavente-García and Castillo, 2008). The flavonoids possess an ability to reduce the expression of pro-inflammatory cytokines, modulate epigenetic control, down-regulate inflammatory biomarkers, and prevent neural damage and many other diseases, mainly due to their potent antioxidant properties (Lee et al., 2009; Almeida Rezende et al., 2016; Hua et al., 2016; Fernandes et al., 2017; Qadir, 2017; Spagnuolo et al., 2018). All of these features of flavonoids make them a promising therapeutic intervention against neurodegenerative diseases. Though several natural products have been shown to have potential epigenetic modulatory properties against cancer and CVDs, very few natural product inhibitors have been shown to modulate the epigenetic pathways in neurological disorders.

Quercetin is a plant flavonoid present in most plants and foods, such as red wine, onions, green tea, apples, berries, Ginkgo biloba, American elder, and others. The molecular mechanisms underlying the neuroprotective actions of quercetin include possible up- and/or down-regulation of cytokines *via* nuclear factor (Nrf2), Paraoxonase-2, c-Jun N-terminal kinase (JNK), Protein kinase C, Mitogen-activated protein kinase (MAPK) signaling cascades, and PI3K/Akt pathways, as demonstrated by *in vivo* and *in vitro* studies (Zaplatic et al., 2019). Cocoa is a rich source of plant flavonoids and shows neuroprotective action against cognitive decline in healthy individuals (Sorond et al., 2008; Lamport et al., 2015). In a clinical trial involving 531 participants aged $\geq$65 years, chocolate consumption for 48 months was associated with a 41% lower risk of cognitive decline (Moreira et al., 2016). Anthocyanin is a bioactive compound found in the seed coat of the black soybean and is reported to inhibit several diseases. A recent study

established that supplementation with anthocyanins mitigates oxidative stress, neurodegeneration, and memory impairment in a mouse model of AD *via* the PI3K/Akt/Nrf2/HO-1 pathways (Ali et al., 2018).

Caffeine reverses cognitive impairment and decreases brain Aβ levels in aged APP mice (Azam et al., 2003; Arendash et al., 2009). This reduction in Aβ plaque might be due to the stimulation of protein kinase A activity, increased phosphor-CREB levels, and reduced phosphor-JNK and phosphor-ERK expression in mouse models of AD and promotes survival cascades in the brain (Zeitlin et al., 2011). Interestingly, higher blood caffeine levels in MCI patients have been linked to a lack of progression to dementia (Cao et al., 2012). A population-based study reported that drinking of 3–5 cups of coffee per day might reduce the incidence of AD and dementia by 65% (Eskelinen et al., 2009). While animal data recommend a protective effect for caffeine on cognition, studies in humans remain inconsistent. A study on 3,494 men showed that coffee and caffeine intake in midlife was not related to cognitive impairment (Gelber et al., 2011). Conflicting results were reported in different populations, wherein a Portuguese study showed an association of caffeine consumption with reduced cognitive decline (Santos et al., 2010), while another study did not show any association in a population in France (Ritchie et al., 2007).

Resveratrol

Resveratrol, a polyphenol present in grapes and red wine, is receiving increasing attention due to its strong antioxidant and anti-inflammatory actions (Gambini et al., 2015; Sawda et al., 2017). Resveratrol exhibits these properties due to its molecular structure, which endows it with the ability to bind with several biomolecules. Resveratrol is known to activate sirtuin 1 (SIRT1), a class III HDAC (Baur, 2010), and thereby protect cells against the inflammation and oxidative damage induced by ROS (Cantó et al., 2009). Resveratrol activates a transcriptional coactivator, PGC-1α, that promotes energy metabolism by glucose uptake and mitochondrial biogenesis (Lagouge et al., 2006; Kumar and Lombard, 2015; Parihar et al., 2015). Recent studies have demonstrated that maternal resveratrol supplementation and vitamin D combined with resveratrol could prevent cognitive impairment in SAMP8 mice offspring through amyloidogenic pathways, neuroinflammation, tau phosphorylation, epigenetic changes, and cell signaling pathways (Cheng et al., 2017; Izquierdo et al., 2019). Another study indicated the ameliorative action of resveratrol in hippocampal neurodegeneration and memory performance (Gomes et al., 2018). Some clinical trials on supplementation with resveratrol for a longer period reported improved cognitive decline and improved functional connectivity of the hippocampus (Witte et al., 2014). Several clinical trials on resveratrol supplementation and its possible neuroprotective impact on cognitive decline, MCI, and AD are ongoing (Tome-Carneiro et al., 2013). Owing to its strong antioxidant, anti-inflammatory, and neuroprotective properties, supplementation with resveratrol may be a promising therapeutic measure to combat the rising prevalence of cognitive deficit and AD (Cheng et al., 2017).

All of these dietary bioactive compounds, such as curcumin, resveratrol, epigallocatechin-3-gallate, genistein, phenylisothiocyanate, and indole-3-carbinol, have the ability to modulate epigenetic mechanisms including regulation of HDAC and HAT activities and acetylation of histones and non-histone chromatin protein (Vahid et al., 2015).

Minerals

Deficiency of dietary minerals such as calcium, magnesium, and potassium plays an important role in a wide variety of critical cellular processes associated with cognitive impairment and dementia (Ozawa et al., 2012; Cherbuin et al., 2014). Substantial evidence shows that higher levels of dietary minerals play a protective role against many metabolic diseases including type 2 diabetes, hypertension, stroke, and cognitive decline (Iso et al., 1999; Larsson and Wolk, 2007; Villegas et al., 2009; Barbagallo et al., 2011). Compelling evidence shows that magnesium deficiency may induce oxidative stress in various tissues through a substantial increase in the formation of free radicals by inflammatory cells, which further impairs memory and contributes to AD pathology (Durlach, 1990; Bardgett et al., 2005; Vural et al., 2010; Barbagallo et al., 2011). Previous studies have demonstrated that magnesium supplementation modifies AβPP processing and stimulates the α-secretase cleavage pathway (Yu et al., 2010) and plays a potential protective role in cognitive dysfunction (Cilliler et al., 2007). Further well-designed clinical trial studies are required to ascertain the protective role of magnesium in cognitive decline and AD pathology.

Vitamin Supplementation

Vitamins perform vital functions in the nervous system and might be useful in maintaining cognitive function and delaying the onset of AD (McCleery et al., 2018). Vitamin supplements are found to be very effective in reducing the burden of chronic diseases, including CVD and cancer. These dietary interventions target various molecular mechanisms in disease pathology, including oxidative stress, mitochondrial dysfunction, inflammatory pathways, and calcium homeostasis, in many diseases. A recent study demonstrated the role of vitamins in aging, MCI, and AD by the modulation of many molecular mechanisms involved in the pathogenesis of the disease (Fenech, 2017). Very few randomized clinical trials have examined the effectiveness of vitamin supplements on the primary prevention of cognitive decline and AD, and contradictory results have been reported from the few clinical studies on dietary interventions in AD. A randomized trial of beta-carotene supplementation and cognitive function in 4,052 men did not show any significant effect on cognitive function (Grodstein et al., 2007). However, mixed results have been reported for vitamin B supplementation in cognitive impairment. A randomized clinical trial of folic acid, vitamin B6, and B12 supplementation by 299 men aged >75 years did not show a significant effect on cognitive function (Ford et al., 2010). Similarly, a meta-analysis of nine RCTs involving 2,835 persons exhibited no significant effect of folic acid with or without other B vitamins on cognitive function (Wald et al., 2010). On the other hand, another study showed that supplementation with folic acid and vitamin

B12 together were significantly improving cognitive functions (Walker et al., 2012).

Mitochondria-Targeted Antioxidants

The excess of ROS produced in cognitive decline and AD is associated with mitochondrial dysfunction, represented by altered biogenesis and dynamics (Calkins et al., 2011). Mitochondria-targeted drugs may be a promising therapeutic strategy in aging and neurodegenerative diseases (Reddy, 2008). In the past decade, many mitochondria-targeted antioxidants have come on the market as supplements for delaying the onset of brain diseases by boosting mitochondrial biogenesis and bioenergetics. These mitochondria-targeted antioxidants are known to improve a variety of pathologic conditions, including heart disease, obesity, diabetes-related complications, and AD by modulating the oxidative stress markers and misfolded proteins (Manczak et al., 2010; Reddy and Reddy, 2011; Bhatti et al., 2017a; Reddy et al., 2017). Some of the mitochondria-targeted antioxidant molecules currently available in the market are MitoQ, MitoVitE, MitoTempo, MitoPBN, and MCAT, which have the potential to limit free radical formation and improve mitochondrial dysfunction in many diseases.

Mediterranean Diet Pattern

Lifestyle and diet have been identified as major risk factors in a number of diseases. The MD, broadly accepted as a healthy eating model, is characterized by the high consumption of plant-based foods, olive oil as the main source of fat, low-to-moderate consumption of fish, dairy products, and poultry, low consumption of red and processed meat, and low-to-moderate consumption of wine with meals. Earlier studies demonstrated that MD is linked with low morbidity and mortality in several diseases including CVDs, diabetes, obesity, cancer, and neurodegenerative diseases (Roman et al., 2008; Temple et al., 2019; Witlox et al., 2019). These dietary interventions impact several cardiovascular risk determinants, including body weight, blood pressure, and lipid levels (Rees et al., 2019; Temple et al., 2019). Previous studies have shown that higher adherence to the MD may reduce the risk of developing diabetes and CVDs (Esposito et al., 2017). The modulatory action of the MD is mediated through the molecular mechanisms involving inflammation and metabolic abnormalities in AD pathology (Akiyama et al., 2000; Esposito et al., 2004; Scarmeas et al., 2006; Gu et al., 2010). Diet-derived bioactive components modulate DNA methylation by altering histones and chromatin structure (Bassett and Barnett, 2014). Recent studies also suggest that following the Mediterranean dietary pattern may reduce the risk of many types of cancers (Farinetti et al., 2017; Jones et al., 2017; Schwingshackl et al., 2017).

LIFESTYLE MODIFICATIONS

Physical Activity

A sedentary lifestyle is considered as one of the risk factors for a wide variety of diseases in the 21st century (Blair, 2009). Physical activity is defined as any bodily movement produced by skeletal muscles that result in energy expenditure (Caspersen et al., 1985). Physically active individuals are healthy and free from many diseases (Colberg et al., 2016). Recent studies demonstrated relative reductions of 10% per decade in the prevalence of seven modifiable risk factors per decade might reduce the prevalence of AD in 2050 by 8.3% worldwide (Norton et al., 2014; Luck and Riedel-Heller, 2016). This kind of preventive measure could have a high impact on the burden of lifestyle-related diseases (Ashby-Mitchell et al., 2017). Physical activity is one of the potentially effective training interventions that can limit the prevalence of a wide variety of cardiometabolic and neurodegenerative diseases by reducing mitochondrial dysfunction by activating various transcription factors in bioenergetics processes (Barbieri et al., 2015).

Regular exercise activates various cell signaling pathways and helps improve the mitochondrial health in the skeletal muscles (Russell et al., 2014). It is known to control the blood sugar level and body weight, maintain blood pressure, reduce dyslipidemia, and improve muscles and bone health. Another study demonstrated a reduction in cognitive decline and a decrease in the accumulation of misfolded proteins in the brain of transgenic animals (Pietropaolo et al., 2008). Another study indicated that physical exercise induces neuroplasticity of the brain and improves cognitive functions, as evidenced by animal and human studies (Hötting and Röder, 2013). Physical activity controls the cellular energy homeostasis through PGC-1α and a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase, SIRT1 (Rodgers et al., 2005). CR or exercise reduces energy and increases the AMP/ATP ratio, which activates 5′-adenosine monophosphate-activated protein kinase (AMPK) in the cells. These events further cause stimulation of a transcription factor, PGC 1, through phosphorylation and then ultimately induce mitochondrial biogenesis (Jäger et al., 2007). With aging, there is a loss of muscle mass and muscle activity. Regular exercise reduces the development of aging-related muscle deterioration and promotes healthy aging (Cartee et al., 2016).

Calorie Restriction

Calorie restriction (CR) is another potentially promising non-pharmacologic intervention that is effective in brain aging by improving metabolic health (Wahl et al., 2019). CR is effective through neutralizing the harmful effects of ROS and oxidative damage (Barja and Herrero, 2000; Zainal et al., 2000; Barja, 2002; Civitarese et al., 2007). CR has been shown to prevent the development of various diseases through sirtuins as a target. A previous study showed that long-term CR significantly reduces β-amyloid and γ-secretase in female Tg2576 mice (Schafer et al., 2015) and plays a preventive role in AD pathology. Observational trials and RCTs indicate that CR in humans improves multiple metabolic factors that are involved in the pathophysiology of cardiometabolic disorders (Fontana, 2008). CR exerts these modulations by enhancing their properties by inhibiting vital nutrient-sensing and inflammatory pathways (Most et al., 2017). So, as well as physical activity and exercise, CR may also be considered as a promising nutritional intervention for the prevention of many age-related chronic diseases.

CONCLUSIONS AND FUTURE PERSPECTIVES

The world population is aging at a greater pace, and age-associated diseases are therefore a matter of concern today. The rapid increase in the incidence and prevalence of dementia globally is a major problem and is linked with social and economic burdens. Unfortunately, there is no permanent cure available to date. Several pharmacological therapeutics targeting the major molecular mechanisms, including Aβ and tau proteins, have failed to achieve satisfactory results in human clinical trials. There is thus an urgent need to slow down cognitive decline and halt the progression to AD. Non-pharmacologic interventions such as lifestyle and nutritional therapies, which have been proved to be beneficial in the many aging-related metabolic diseases including diabetes, obesity, CVD, and cancer, may help in the prevention of dementia. The unsatisfactory results of some of the clinical trials may be due to methodological heterogeneity among different studies. These non-pharmacologic interventions, if implemented carefully, may be very effective in reducing the exponential rise in the incidence of AD and curtailing the economic burden on the individuals affected and society. The area that needs great attention is the molecular mechanisms and effective therapeutic targets for AD. Also, large randomized clinical trials should be carried out to ascertain the effectiveness of new drugs or non-pharmacologic interventions for cognitive deficit and AD in the human population. The next decade will be a critical period for the scientific community to discover an effective treatment for AD.

AUTHOR CONTRIBUTIONS

GB, JB, AR, and PR contributed for planning, execution, writing and final drafting of the manuscript.

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